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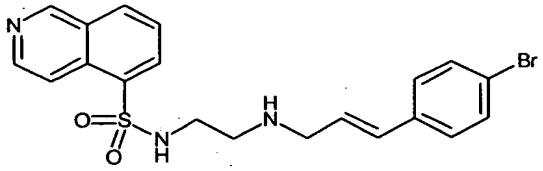

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Extracellular Signal-Regulated Kinases Regulate Dendritic Growth in Rat Sympathetic Neurons.

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Abstract

NGF activates several signaling cascades in sympathetic neurons. We have examined how activation of one of these cascades, the ERK/MAP kinase pathway, affects dendritic growth in these cells. Dendritic growth was induced by exposure to NGF and BMP-7. Exposure to NGF increased phosphorylation of ERK1/2. Unexpectedly two MEK inhibitors (PD 98059 and U 0126) enhanced dendritic growth and a ligand, basic FGF, that activates the ERK pathway inhibited the growth of these processes. The enhancement of dendritic growth by PD 98059 was associated with an increase in the number of axo-dendritic synapses and it appeared to represent a specific morphogenic effect because neither axonal growth nor cell survival was affected. In addition, increased dendritic growth was not observed following exposure to inhibitors of other signaling pathways, including the phosphatidylinositol-3-kinase inhibitor LY 294002. Dendritic growth was also increased in cells transfected with dominant negative mutants of MEK1 and ERK2, but not with dominant negative mutants of MEK5 and ERK5, suggesting that ERK1/2 is the primary mediator of this effect. Exposure to BMP-7 induces nuclear translocation of SMAD1 and PD 98059 treatment potentiated nuclear accumulation of Smad-1 induced by BMP-7 in sympathetic neurons, suggesting a direct enhancement of BMP signaling in cells treated with a MEK inhibitor. These observations indicate that one of the signaling cascades activated by NGF can act in antagonistic manner in sympathetic neurons and reduce the dendritic growth induced by other NGF-sensitive pathways.

Introduction

Dendrites are the primary site of synapse formation in the vertebrate nervous system. Therefore, it is important to understand how the growth of these processes is regulated. Many growth factors have been found to stimulate dendritic growth (Higgins et al., 1997) but two families appear to have especially prominent and widespread effects. Members of the bone morphogenetic protein (BMP) family enhance dendritic growth in sympathetic, spinal motor, cortical, striatal, and hippocampal neurons (Lein et al., 1995; Withers et al., 2000; Esquenazi et al., 2002; Gratacos et al., 2002) whereas various neurotrophins increase the growth of these processes in cortex (McAllister et al., 1995; Baker et al, 1998), retina (Lom et al., 2002) and cerebellum (Schwartz et al., 1997). Moreover, there is evidence for interactions between these two classes of stimulatory molecules with sympathetic neurons requiring simultaneous exposure to both a member of the BMP family and to nerve growth factor (NGF) for optimal dendritic growth (Lein et al., 1995). A variety of cytokines and hormones have also been found to inhibit dendritic growth (Guo et al., 1999; Chandrasekaran et al., 2000; Drahushuk et al., 2002; Kim et al., 2002). However, the signaling cascades triggered by these stimulatory and inhibitory agents and their interactions remain poorly characterized.

The binding of neurotrophins to trk receptors activates several signaling pathways. These include the extracellular signal-regulated kinase (ERK) cascade, phosphatidylinositol-3-kinase (PI-3 kinase)/Akt kinase, and phospholipase C (PLC)-γ1 (Huang and Reichardt, 2001; Chao, 2003). Of particular interest is the ERK pathway, which has many effects on neural tissues. For example, activation of ERKs increases axonal growth in sympathetic neurons (Atwal et al., 2000), dorsal root ganglia (Sjogreen et al., 2000; Wiklund et al., 2002), and PC12 cells (Traverse et al., 1992; Pang et al., 1995) in vitro and it also enhances axonal regeneration

following axotomy in vivo (Miura et al., 2000). In addition, ERK stimulation plays an important role in activity-dependent regulation of neuronal functions such as synaptic plasticity, learning, and memory (Grewal et al., 1999; Adams and Sweatt, 2002). In this study we examined the role of the ERK pathway in the regulation of dendritic growth in sympathetic neurons exposed to BMP-7 and NGF. Co-stimulation with these two agents leads to a rate of dendritic growth in vitro that is equivalent to that observed in vivo (Lein et al., 1995) and there is evidence for sympathetic neurons being exposed to both of these growth factors in vivo, with NGF being derived from target tissues (Purves et al., 1988) and BMPs being produced by glia (Lein et al., 2002).

Our data indicate that inhibition of ERK1/2 activation by either pharmacological agents or overexpression of dominant-negative mutants potentiates dendritic growth in the presence of BMPs and NGF and that this represents a specific morphogenetic effect because neither axonal growth nor cell survival is affected. These observations indicate that one of the signaling cascades activated by NGF acts in antagonistic manner in sympathetic neurons and reduces the dendritic growth induced by other NGF-sensitive signaling pathways.

Materials and Methods

Materials

Recombinant human BMP-2, BMP-6 and BMP-7 were generous gifts from Curis (Cambridge, MA). PD 98059 was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA) and U0126 was from Promega (Madison, WI). LY 294002 was obtained from Sigma (St. Louis, MO) and SB 202190 from Calbiochem (San Diego, CA).

Cell Culture

Cultures of neurons from the superior cervical ganglia of embryonic (day 21) Holtzman rats (Harlan Sprague-Dawley, Rockford, IL) were prepared according to previously described methods (Higgins et al., 1991). Briefly, cells were dissociated following enzymatic treatment with trypsin (2.5 mg/ml) and collagenase (1 mg/ml). Subsequently they were pelleted, resuspended in serum-free medium and plated at low density (~10 cells/mm^2) onto poly-D-lysine (100 μg/ml) coated coverslips. The serum-free culture medium (Higgins et al., 1991) contained β-nerve growth factor (NGF, 100 ng/ml). To kill dividing non-neuronal cells, the anti-mitotic agent cytosine-β-D-arabinofuranoside (1 μM) was added to the medium for 48 hr beginning on day 2. Experimental treatments were begun on the 6th or 7th day in vitro.

Morphological analyses

Cellular morphology was analyzed by immunocytochemical methods (Lein et al., 1995). Culture were fixed with 4 % paraformaldehyde and permeabilized with 0.1 % Triton- X 100 in PBS (3 min). Cells were then incubated with monoclonal antibodies (mAb) that recognize either

axons or dendrites, followed by detection with rhodamine conjugated secondary antisera (Roche Diagnosis Co., Indianapolis, IN). A mAb to microtubule-associated protein-2 (MAP2, SMI-52; Sternberger Immunocytochemicals, Baltimore, MD) was used to visualize dendrites. Axons were identified with a mAb to phosphorylated forms of the H and M neurofilament subunits (SMI-31; Sternberger Immunocytochemicals). SPOT software (Diagnostic Instruments Inc., Sterling Heights, MI) was used to measure the total neuritic length. Synaptic specializations that formed along dendrites were visualized by double-labeling the cultures with rabbit anti-MAP2 IgG (a gift from Dr. Craig Garner, University of Alabama at Birmingham, Birmingham, AL) and mAb to synaptic vesicle protein-2 (SV-2; Developmental Hybridoma Bank, University of Iowa) (Feany *et al.*, 1993) and then with rhodamine conjugated antibody to rabbit IgG and fluorescein conjugated antibody to mouse IgG (Roche Diagnostics Co., Indianapolis, IN).

Axonal growth was also assessed by plating cells at low density (~ 3,000 cells/coverslip) onto gridded coverslips with embossed coordinates (CELLocate, Eppendorf Scientific, Inc, Hamburg, Germany). Under these conditions, one can serially monitor axonal growth for extended periods by relocating previously examined neurons (Guo et al., 1999). After eliminating non-neuronal cells, neurons were grown in medium with or without PD 98059 for 5 days and total axonal length was periodically measured with NIH Image J (1.25s).

The rabbit IgG fraction used to examine the cellular distribution of Smad-1 was obtained from Upstate Biotechnology (Lake Placid, NY). Briefly, cultures were fixed with 4 % paraformaldehyde (15 min, 20°C) and permeabilized with methanol (10 min, - 20°C). Cells were examined with a Bio-Rad confocal microscope, using 1 μm optical sections.

Experiments were typically performed 3 times and data are presented as the mean ± S.E.M.. Statistical significance was assessed by Student's *t*-test or a one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test.

Western blotting

Cells were solubilized in 50 mM Tris-HCl (pH 7.4), 1mM EDTA, 0.1 % SDS and 2.0 % β-mercaptoethanol. After centrifugation at 15,000 X g for 10 min, the protein concentration was determined using a Bio-Rad protein assay. Proteins (10 μg/lane) were separated by SDS-PAGE (12 %) and transferred to PVDF membranes. Blots were probed with polyclonal antibodies to ERK1/2 or phosphorylated-ERK1/2 (Cell Signaling, Beverly, MA) or a mAb to MAP2 (SMI 52) and incubated with an HRP-conjugated secondary antibodies (Boehringer Mannheim Biochemicals, Indianapolis, IN). Detection was performed using an enhanced chemiluminescent reagent (Amersham, Piscataway, NJ). To quantify data, films were scanned using an HP ScanJet ADF scanner and HP Precision ScanPro software, and band density determined as arbitrary absorption units.

Transfection

Cells were cotransfected with a plasmid encoding the enhanced green fluorescent protein (pEGFP-N2, Clontech, Palo Alto, CA) and plasmids containing either MEK or ERK constructs. The former were a generous gift from Dr. Natalie Ahn (University of Colorado, Boulder, Colorado) and consisted of the expression vector pMCL containing HA-MAPKK (wild type), HA-K97M MAPKK(dominant negative carrying M substitution at K97), or HA-S218E/S222D MAPKK (constitutively active) (Mansour et al., 1994). Dominant-negative ERK2, ERK5, and

MEK5 constructs (Kato et al., 1997) were kindly provided by Dr. Lee (Scripps Research Institute, La Jolla). Transfection was performed using LipofectAmine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Briefly, cells in 12 well dishes were treated on day 5 with 500 μl/well of Dulbecco's modified Eagle's medium containing 1.5 μg of DNA and 6 μg of LipofectAmine. After incubation for 6 hr, cells were washed and allowed to recover for 48 hr before experimental treatments were begun. Transfection efficiency was typically ~20% (Kim et al., 2002)

Results

Inhibition of MEK enhances dendritic growth and synapse formation.

Embryonic sympathetic neurons extend only axons (Bruckenstein and Higgins, 1988a) when grown in serum-free medium in the absence of non-neuronal cells (Fig. 1). Exposure to a modest concentration (10 ng/ml) of BMP-7 causes these neurons to form 2-3 dendrites over 5 days (Fig. 1). Neurons treated with maximally effective doses ($\geq$ 50 ng/ml) of BMP-7 extend a greater number of primary dendrites (Fig. 2) and eventually generate a dendritic arbor that is equivalent in size to that observed in situ (Lein et al., 1995).

We wanted to examine the possibility that the MAPK pathway regulates dendritic growth. Sympathetic neurons were grown in the presence of a maximally effective concentration (100 ng/ml) of NGF. Therefore, it was expected that the MEK/MAPK pathway would be active under our culture conditions (Creedon et al., 1996). To assess morphological changes in dendrites, cells were exposed to an inhibitor of MEK1 (PD 98059) with or without BMP-7 (10 ng/ml) for 5 days. PD 98059 alone did not induce morphological changes in sympathetic neurons (Fig. 1). However, it potentiated the inductive effects of BMP-7 on dendritic growth. The potentiating effects of PD 98059 were detected at concentrations as low as 0.1 μM and the ED_{50} was ~ 0.3 μM (Fig. 2). In cultures treated with moderate doses of BMP-7 (10 ng/ml), PD 98059 produced a 2-fold increase in the number of dendrites and total dendritic length increased 3-fold (Fig.2). The potentiating effects of PD 98059 were also observed in the presence of maximally effective concentrations of BMP7 (> 50 ng/ml): there was a ~50% increase in the number of dendrites and a ~35% in total dendritic length. Non-linear regression analysis (Marquardt-Levenberg

algorithm) revealed that PD 98059 increased the maximum response, i.e., it increased the number of dendrites that sympathetic neurons develop in culture but did not affect the ED_{50}. These data suggest that the effect of PD 98059 on BMP7-induced dendritic growth is an enhancement of BMP7 efficacy. PD 98059 also enhanced BMP-2 and BMP-6-induced dendritic growth (data not shown), suggesting that PD 98059 affects a common pathway in BMP signaling.

To further characterize the effects of MEK inhibition on sympathetic neurons, we examined the effects of PD 98059 on MAP2 expression and synapse formation. MAP2 is a protein that is found primarily dendrites and its expression increases following exposure to BMP-7 (Guo et al., 1998). In agreement with previous reports, Western blotting revealed that the expression of MAP2 increased 2.8 ± 0.2 fold after 48 hours of BMP-7 exposure. In the presence of PD 98059, the increase in MAP2 expression was greater (3.5 ± 0.1 fold increase), suggesting a modest synergistic action on MAP2 expression. Treatment with PD 98059 also increased the formation of axo-dendritic synapses by 46% (Fig. 3). Thus MEK inhibition not only enhances the growth of dendrites, but also increases the number of contacts made with these processes.

To confirm that MEK was indeed active in our system and that PD 98059 blocked its activity, we examined the activation of ERK1/2, which are kinases downstream from MEK. Western blotting was performed, using an antibody that recognizes a phosphorylated form of ERK1/2 (Thr 202/Tyr 204). Pretreatment of cells with PD 98059 (10 μM) for 30 min completely inhibited the phosphorylation of ERK1/2 produced by NGF (100 ng/ml) (not shown). The specificity of action of PD 98059 was further assessed by comparing its effects with those of a structurally unrelated MEK inhibitor U 0126 (Fig. 4). A 2.5-fold increase in the number of dendrites per cell was observed in cultures treated with U 0126 in the presence of BMP-7,

strongly suggesting that the potentiation of dendritic growth by PD 98059 is due to its ability to inhibit MEK.

We next determined whether a dominant-negative mutant of MEK1 that is catalytically inactive (K97M) also enhances BMP-7-induced dendritic growth. For these transfection experiments, we needed to use higher density cultures in which dendritic growth was less robust than in low density cultures used in other experiments (Fig 5). However, inhibition of MEK also enhanced dendritic growth in these cultures (not shown). Wild type or mutant MEK1 was cotransfected with EGFP to identify the transfected cells. Forty-eight hours later, cells were treated with BMP-7 (10 ng/ml) for 5 days. In cultures transfected with control vector or wild type MEK1, BMP-7 caused cells to extend ~1 dendrite, indicating that transfected cells responded to BMP-7 (Fig. 5). Consistent with previous results, overexpression of the dominant-negative mutant of MEK1 potentiated BMP-7-induced dendritic growth by ~1.8-fold. Moreover, constitutively active MEK1 decreased dendritic growth, strongly suggesting that the ERK/MAPK pathway regulates dendritic growth in sympathetic neurons.

NGF causes activation of both ERK1/2 and ERK5 in sympathetic neurons and there is evidence indicating the various ERKs subserve different cellular functions in these cells (Watson et al., 2001). To determine whether a particular isoform was involved in the regulation of dendritic growth, sympathetic neurons were transfected with dominant negative constructs of ERK2 and ERK5. Only the former enhanced dendritic growth (Fig 6). Dominant negative mutants of ERK5 and MEK5, the kinase upstream of ERK5, were inactive.

MAP kinase does not affect cell number or axonal growth.

To determine whether PD 98059 selectively affects dendrites, we examined its effects on cell survival. There was no significant change in neuronal number in the presence of a maximally effective concentration (10 μM) of PD 98059 (Fig. 7). This finding is consistent with previous reports that inhibition of MEK activity by PD 98059 does not suppress the survival of sympathetic neurons (Creedon et al., 1996; Virdee and Tolkovsky, 1996).

Inhibition of MEK activation inhibits neuritic growth in PC12 cells (Cowley et al., 1994; Pang et al., 1995) and axonal regeneration after axotomy in sensory neurons of the dorsal root ganglia (Sjogreen et al., 2000; Wiklund et al., 20002). Therefore, we used 2 methods to determine whether PD 98059 affects axonal growth in our system (Fig. 8). First, initial axonal outgrowth was assessed at 12 hrs after plating. Immuostaining with an antibody that recognizes the phosphorylated forms of the H and M neurofilament subunits showed that PD 98059 does not interfere with initial axonal growth. To examine possible long time effects of PD 98059 on axonal growth, neurons were plated onto gridded coverslips at low density, which allowed us to observe morphological changes of individual axons in the same region over the time (Fig. 8). Treatment with PD 98059 began on day 5 *in vitro*, at which time a minimal level of axonal growth had been established. Total axonal length was reassessed 1, 3, and 5 days later and serial imaging showed that the inhibition of MEK activity does not affect axonal growth. These data suggest that MAP kinase selectively affects dendritic growth in sympathetic neurons grown under serum-free conditions.

Comparison of effects of MEK inhibitors and other kinase inhibitors.

The p38 kinase, a member of the MAPK subfamily, has also been implicated in neuronal differentiation, including NGF-induced neuritic growth of PC12 cells (Morooka and Nishida,

1998; Hansen et al., 2000). In addition, PI-3 kinase is activated by neurotrophic factors that regulate the axonal growth and survival of sympathetic neurons (Kuruvilla et al., 2000). To determine if these kinases regulate dendritic growth, sympathetic neurons were exposed to SB 203580, a p38 kinase inhibitor, or the PI-3 kinase inhibitor, LY294002, in the presence of BMP-7 (10 ng/ml) for 6 days (Table 1). Neither inhibitor potentiated BMP7- induced dendritic growth. The inhibition of dendritic growth by LY 294002 may be due to its deleterious effects on sympathetic neuronal health survival (Crowder and Freeman, 1998; Mazzoni et al., 1999) and somatic atrophy was observed in our cultures.

FGF inhibits BMP-7-induced dendritic growth

We next determined whether other growth factors that activate the ERK pathway also inhibit BMP-7-induced dendritic growth. Neurons were cultured in the media containing 10 ng/ml of NGF. This concentration of NGF allows high rates of survival (90-100%) of sympathetic neurons (Belliveau et al., 1997). However, it is suboptimal for promoting dendritic growth and thus provides favorable conditions for examining effects of FGF-induced ERK activation on dendritic growth. In cultures treated with moderate doses of BMP-7 (5 ng/ml), there was a 45 % decrease in the number of dendrites/ cell in the presence of FGF (Fig. 9), suggesting that FGF-induced ERK activation inhibits dendritic growth in sympathetic neurons.

MAPK blocks the nuclear accumulation of Smad-1.

MAPK has been shown to inhibit the nuclear accumulation of Smad-1 by phosphorylating a site in the linker region between DNA-binding domain and the transcriptional activation domain (Kretzschmar et al., 1997). To explore the mechanism involved in MAPK-mediated regulation

of dendritic growth, we examined the possibility that the inhibition of MAPK activity enhances nuclear accumulation of Smad-1. Treatment with a maximally effective concentration of BMP-7 (100 ng/ml) caused marked induction and nuclear translocation of Smad-1 within 2 hr (~ 90 % nuclei labeled; Fig. 10). In contrast, treatment of BMP-7 at a concentration approximating the EC_{50} (10 ng/ml) did not cause a detectable effect at this time; most cells still showed modest Smad-1 staining in the cytoplasm (~ 15 % nuclei labeled). These data indicate that lower concentrations of agonist cause a decrease in the kinetics of signaling events mediated by BMP-7. However, PD 98059 treatment potentiated the induction and nuclear accumulation of Smad-1 (~ 42 % nuclei labeled), as compared to treatment with the lower concentration of BMP-7 alone.

Discussion

The ERK signaling pathway regulates crucial aspects of neuronal development, including cellular survival and differentiation (Traverse et al., 1992; Pang et al., 1995; Bergmann et al., 1998; Kurada and White, 1998; Anderson and Tolkovsky, 1999; Atwal et al., 2000; Wiklund et al., 2002). Activation of the ERK signaling pathway has also been found to alter axonal growth in sympathetic, sensory and spinal motor neurons (Atwal et al., 2000; Miura et al., 2000; Sjogreen et al., 2000; Wiklund et al., 2002) and neurite outgrowth in PC 12 cells (Traverse et al., 1992; Pang et al., 1995). In most instances the effects of ERK activation on axonal growth have been stimulatory. In contrast, our data indicate that inhibition of ERK signaling enhances BMP-7-induced dendritic growth and synapse formation, indicating that activation of this pathway may adversely affect neural development under some circumstances.

Our observations contrast with a recent reporting suggesting that ERKs mediate activity-dependent increases in dendritic growth. In particular, Vaillant et al. (2002) found that depolarization of sympathetic neurons via exposure to KCl stimulates the ERK pathway and that this increases dendritic stability. Subtle differences in the culture systems, such as the use of serum (Bruckenstein et al., 1988b) by Vaillant et al., may underlie these discrepant results. However, it should be noted that basal dendritic growth was limited in their control cultures (average total dendritic arbor of ~13 μm) and that KCl provided only a moderate enhancement of dendritic growth (total arbor of ~ 23 μm). In contrast, the amount of dendritic growth in our BMP-7 treated cultures (~300 μm in 5 days) was an order of magnitude greater and indeed the rate of growth was close to that which is observed in vivo (Lein et al., 1995). Thus, while there may be circumstances where depolarization-induced ERK activation can stimulate dendritic

growth such as the refinement of terminal processes, it is likely that opposite effects dominate when there is rapid expansion of the dendritic arbor.

Sympathetic neurons require NGF for their survival (Levi-Montalcini, 1987; Deckwerth and Johnson Jr., 1993). Therefore, most tissue culture media contain a saturating concentration of this trophic factor. In agreement with others (Huang and Reichardt, 2001; Chao, 2003), we found that NGF activates the MEK pathway and thereby causes the phosphorylation of ERK1/2. Surprisingly, two pharmacological inhibitors of the ERK pathway and a dominant negative mutant of MEK1 stimulated dendritic growth, indicating that the effect of ERK activation on dendrites is inhibitory. In contrast, NGF is known to stimulate dendritic growth in vivo and in vitro (Purves et al., 1988; Snider 1988; Lein et al., 1995). This apparent contradiction is probably explained by the fact that NGF activates multiple signal transduction cascades via trkA receptors, including the ras/ERK pathway, PI-3 kinase/Akt kinase pathway, and PLC-γ1 (Segal and Greenberg, 1996; Huang and Reichardt, 2001). Of particular interest is PI-3 kinase/Akt signaling. Since it is required for survival and neuronal hypertrophy (Crowder and Freeman, 1998; Mazzoni et al., 1999; Kuruvilla et al., 2000; Tsui-Pierchala et al., 2000), it seems a reasonable candidate for mediating increased dendritic growth by NGF. Our finding that an inhibitor of the PI-3 kinase pathway inhibits dendritic growth is consistent with this hypothesis. Similarly in a previous study (Drahushuk et al., 2002), we found that an inhibitor of PLC reduced BMP-induced dendritic growth. These data suggest that the net effects of NGF on dendritic growth represent the sum of an inhibitory signaling component via ERK signaling and stimulatory signals from other trk- or p75-mediated signaling pathways. In this respect it is important to note that the MEK-ERK and the PI-3 kinase-Akt pathways are often simultaneously activated in response to growth factors and hormones, and, although the most frequent

interaction between these two pathways is synergistic, there are also many instances (Kauffmann-Zeh et al., 1997; Rommel et al., 1999) in which they have been found to function in an antagonistic manner in non neuronal tissues. For example, inhibition of ILGF-induced ERK activation increases myogenic differentiation whereas inhibition of ILGF-induced Akt activation decreases myogenic differentiation (Coolican et al., 1997). Such opposing interactions offer the potential for fine tuning critical cellular functions such as dendritic growth and synapse formation. Moreover, they allow potential integration of signaling pathways activated by growth factors, transmitters, and activity.

Recent studies suggest that neurotrophins activate different signaling pathways depending on the location of stimulation and that these may have different cellular consequences. Watson et al. (2001) showed that direct application of NGF to the cell body induces phosphorylation and nuclear translocation of both ERK1/2 and ERK5. In contrast, exposure of axon terminals to NGF caused rapid activation of only ERK5 in the cell body. Similarly, the effects of BDNF on dendritic growth in retinal ganglion cells vary depending on its source (Lom and Cohen-Cory, 1999; Lom et al., 2002): dendritic growth is promoted by target-derived BDNF, whereas it is inhibited by locally applied BDNF. Our data indicate the inhibitory effects of ERK on dendritic growth are mediated by ERK1/2 and that ERK5 is not involved, suggesting that it is local ERK activation occurring in the cell body or dendrites rather than axon terminals that is inhibitory for sympathetic neurons. In this respect it is important to note that other activators of the ERK pathway, such as FGF, can also inhibit dendritic growth and that preganglionic sympathetic nerve terminals contain peptides such as PACAP and VIP (Baldwin et al., 1991; Sasek et al., 1991; Beaudet et al., 1998) that are known to cause ERK activation

(Frodin et al., 1994; Young et al., 1994; Barrie et al., 1997) and to inhibit dendritic growth (Drahushuk et al., 2002).

The cellular mechanism by which ERK signaling affects BMP-7-induced dendritic growth may involve interactions with proteins involved in BMP signaling. We found that treatment with PD 98059 enhances the nuclear accumulation of Smad-1 in response to a low concentration of BMP-7 and that it increases nuclear accumulation to a degree comparable to that of cultures treated with a maximally effective concentration of BMP-7. These data suggest that ERKs negatively affect the transcriptional activity of Smad-1. Similarly, Kretzchmar et al (1997) reported that activation of the ERK pathway inhibits the nuclear accumulation of Smad-1 by causing phosphorylation of the hinge region linking the inhibitory and effector domains of Smad1. This phosphorylation site is separate and distinct from the C-terminal sequence of Smad-1 phosphorylated by the BMP-7 receptor. However, it plays a critical role in regulating Smad-1 activity (Kretzchmar et al., 1997). In addition, oncogenic Ras represses TGF-β signaling through down-regulation of Smad-4, the common-partner Smad (Saha et al., 2001), which is mediated by the activation of ERK pathway. Thus interactions between ERKs and downstream mediators of BMP signaling may account for some of inhibitory effects of ERK activation on dendritic growth in sympathetic neurons.

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Table 1. Comparison of effects of MEK inhibitors and other kinase inhibitors on BMP-7-induced dendritic growth.

Treatment	Dendrites/ Cell
BMP7	3.21 ± 0.21
BMP7 + PD 98059	5.37 ± 0.22 *
BMP7 + U 0126	5.98 ± 0.24 *
BMP7 + SB 202190	3.38 ± 0.18
BMP7 + LY 294002	1.97 ± 0.14 *

Sympathetic neurons were exposed to BMP7 (10 ng/ml) alone or with one of following kinase inhibitors for 5 d: PD 98059 (10 μM), U 0126 (10 μM), SB 202190 (10 μM) or LY 294002 (10 μM). Dendrites were detected by immunostaining with a mAb to MAP2. * $p < 0.05$ vs. BMP-7.

FIGURE LEGENDS

Figure 1. Effects of BMP-7 and PD 98059 on the morphology of sympathetic neurons. Phase-contrast (A, C, E, G) and fluorescence (B, D, F, H, I, J) micrographs of neurons immunostained with a mAb to MAP2. Neurons in control cultures had only axons (A, B). Neurons exposed to BMP-7 (10 ng/ml) for 5 days extended dendrites (C, D) and this response was enhanced by treatment with PD 98059 (10 μM; E, F). PD 98059 alone had no effect on dendritic growth (G, H). Panels (I) and (J) are low power micrographs of cells exposed to BMP alone (I) or in the presence of PD 98059 (J). Bars, 25 μm.

Figure 2. Inhibition of MEK1 enhances BMP-7-induced dendritic growth. Sympathetic neurons were exposed to various concentrations of the MEK inhibitor PD 98059 (A) with or without BMP-7 (10 ng/ml) for 5 days. Alternatively cells were exposed to varying concentrations of BMP-7 (B, C) in the presence of PD 98059 (10 μM). The number of dendrites/cell (B) and total dendritic length (C) was assessed by immunostaining with mAb to MAP2 ($N \geq 60$ cells). * $p < 0.05$ vs control

Figure 3. Effects of a MEK inhibitor on synapse formation in cultures of sympathetic neurons. Neurons were treated with BMP-7 (10 ng/ml) with or without PD 98059 (10 μM) for 5 days. Dendritic morphology and presynaptic specializations were analyzed by double-immunostaining with antibodies to MAP2 and SV2 ($N \geq 30$ cells/condition). SV2-positive puncta that are associated with dendrites have previously been shown to represent sites of synaptic contact (Fletcher *et al.*, 1991). * $p < 0.05$ vs control.

Figure 4. Effects of U 0126 on dendritic growth. Sympathetic neurons were exposed to various concentrations of the MEK inhibitor U 0126 with or without BMP-7 (10 ng/ml) for 5 days. Cellular morphology ($N \geq 60$ cells) was assessed by immunostaining with mAb to MAP2. * $p < 0.05$ vs BMP-7 alone

Figure 5. Dominant-negative MEK1 potentiates BMP-7-induced dendritic growth. For transfections, sympathetic neurons were plated at three-fold higher density (~30 cells/mm^2) than in previous experiments. Under these conditions, BMP-7 (10 ng/ml) still induced dendritic growth but the magnitude of the response was reduced (A). Neurons were cotransfected with plasmids containing EGFP and wild type MEK1 (MEK1 WT) or dominant negative mutant (MEK1 K97M) (B) or with a constitutively active mutant (D). Two days later, cells were treated with BMP7 (10 ng/ml). On day 5, cellular morphology was assessed by immunostaining with a mAb to MAP2 ($N \geq 60$/group). Transfected cells were identified by expression of EGFP. * $p < 0.05$ vs BMP-7.

Figure 6. Dominant negative ERK2 enhances dendritic growth. Sympathetic neurons were cotransfected with plasmids containing EGFP and one of the following constructs: dominant negative ERK2 (AEF; Thr183and Tyr185 replaced with alanine and phenylalanine; Kato et al., 1997), dominant negative ERK5 (AEF) or dominant negative MEK5 (A). Two days later, cells were treated with BMP7 (10 ng/ml). On day 5, cellular morphology was assessed by immunostaining with a mAb to MAP2. Transfected cells were identified by expression of EGFP. * $p < 0.05$ vs BMP-7.

Figure 7. Inhibition of MEK1 does not alter cell survival.

Sympathetic neurons were treated with BMP-7 (10 ng/ml) with or without PD 98059 (10 μM) for 5 days. Subsequently cell survival was determined by counting the number of MAP2 positive cells.

Figure 8. Inhibition of MEK1 activity does not affect axonal growth.

(A) Dissociated neurons were plated onto coverslips coated with laminin (2 μg/ml) in control medium (C2) or in the presence of PD 98059 (10 μM) and/or BMP-7 (10 ng/ml) for 12 hrs. Axons were identified by immunostaining with a mAb that recognizes phosphorylated forms of H and M neurofilament subunits ($N \geq 40$ cells). (B, C, D) Neurons were plated at low density onto gridded coverslips. After elimination of non-neuronal cells, neurons were grown in medium with or without PD 98059 (10 μM) and/or BMP-7 (10 ng/ml) for 5 days. Representative areas of the cultures were photographed just before treatments and after 1, 3, and 5 days of treatment. The total linear length of all axonal processes with the designated area was determined using NIH J image software. (C) Phase-contrast micrograph of a representative area, taken before experimental treatment was begun. When the same area was relocated after 5 days of treatment with PD 98059, (D) the axonal network had become much denser and new processes had appeared. Circle represents the defined area inside which axons were serially traced; Dark lines indicate alignment reference points for repeated placement of saved circle template. (B) Total axonal length (15 areas).

Figure 9. FGF inhibits BMP-7-induced dendritic growth.

Sympathetic neurons were cultured in the control media containing 10 ng/ml of NGF to maintain cell survival. After elimination of non-neuronal cells, neurons were exposed to BMP7 (5 ng/ml) with or without FGF (100 ng/ml). On day 5, cellular morphology ($N \geq 60$ cells) was assessed by immunostaining using mAb to MAP2. * $p < 0.05$ vs BMP-7.

Figure 10. Inhibition of MEK1 increases the nuclear accumulation of Smad-1.

Sympathetic neurons were cultured under control conditions (A, B) or in the presence of a maximally effective dose of BMP-7 (100 ng/ml; C, D) for 2 hr. In addition, cultures were treated with BMP-7 at a concentration (10 ng/ml) close to ED_{50} in the presence (G, H) or absence of PD 98059 (E, F). PD 98059 (10 μM) was added 30 min before BMP-7 was maintained in the medium thereafter. Cells were immunostained with an antibody that reacts with Smad-1 and optical sections (1 μm) were obtained with a Bio-Rad confocal microscope. Phase-contrast (A, C, E, G) and fluorescence (B, D, F, H) micrographs.

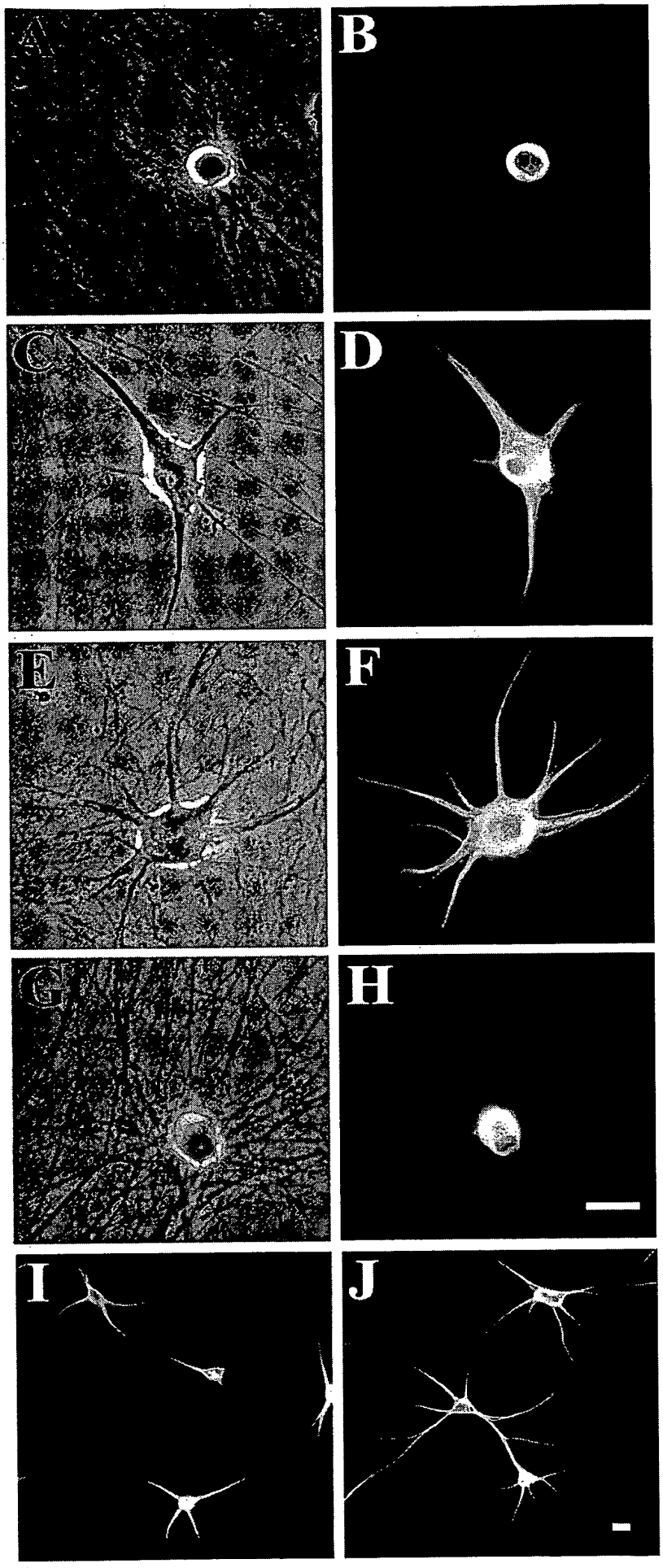


Figure 1

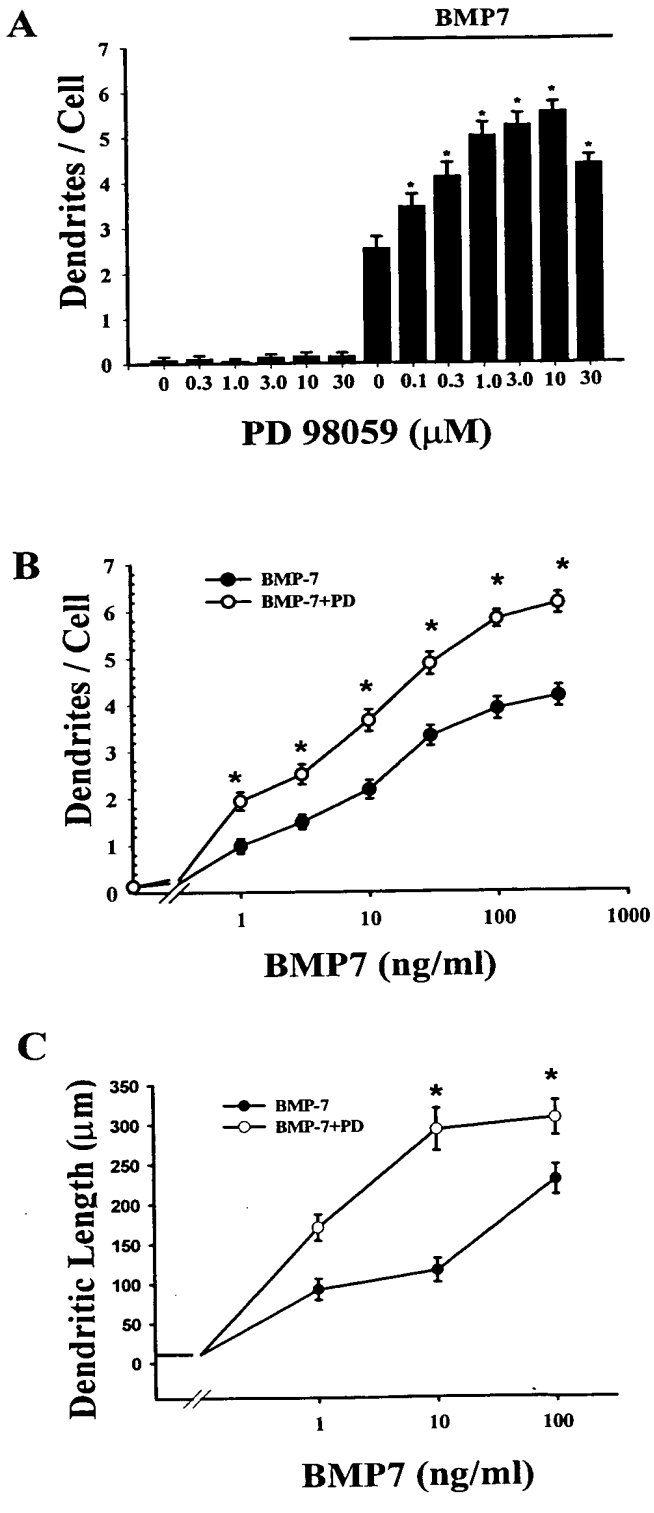

A
BMP7
Dendrites / Cell
0 0.3 1.0 3.0 10 30 0 0.1 0.3 1.0 3.0 10 30
PD 98059 (μM)
B
BMP-7
BMP-7+PD
Dendrites / Cell
1 10 100 1000
BMP7 (ng/ml)
C
BMP-7
BMP-7+PD
Dendritic Length (μm)
1 10 100
BMP7 (ng/ml)

Figure 2

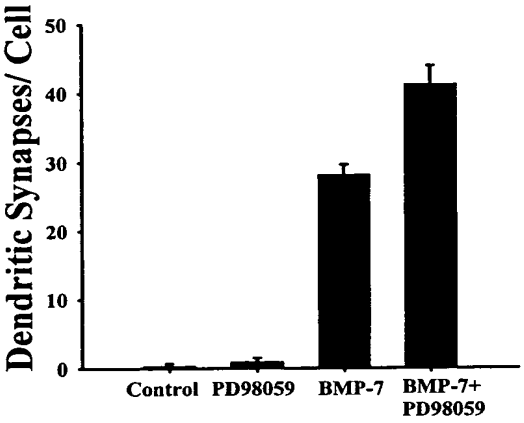

Dendritic Synapses/ Cell
50
40
30
20
10
0
Control
PD98059
BMP-7
BMP-7+
PD98059

Figure 3

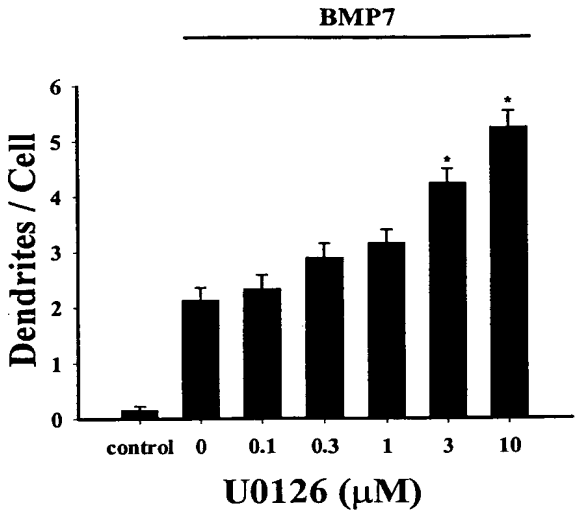


Figure 4

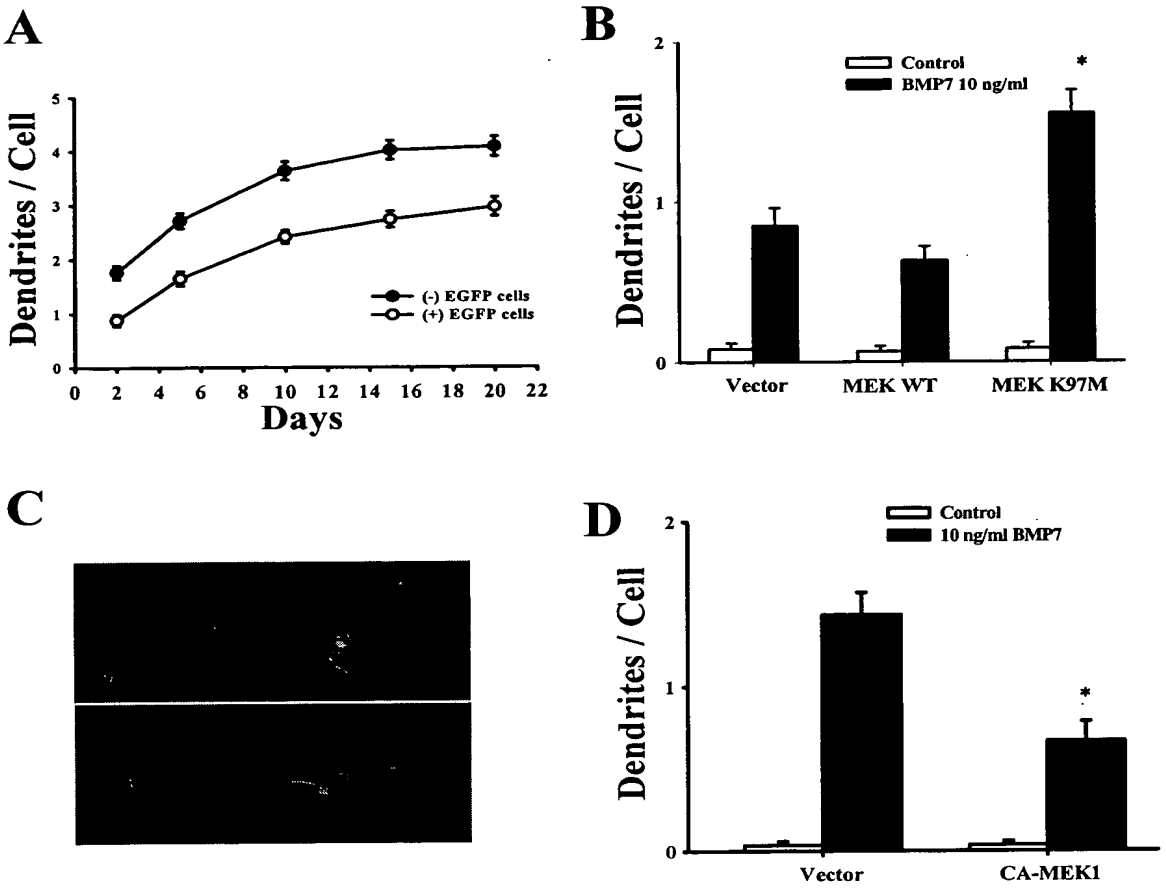

A
Dendrites / Cell
Days
(-) EGFP cells
(+) EGFP cells
B
Dendrites / Cell
Control
BMP7 10 ng/ml
*
Vector
MEK WT
MEK K97M
C
D
Dendrites / Cell
Control
10 ng/ml BMP7
*
Vector
CA-MEK1

Figure5

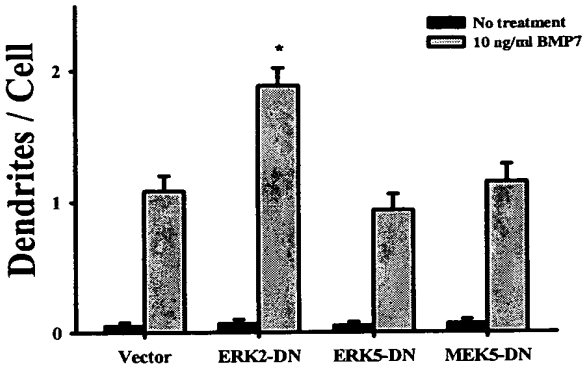

Dendrites / Cell
0
1
2
*
No treatment
10 ng/ml BMP7
Vector
ERK2-DN
ERK5-DN
MEK5-DN

Figure 6

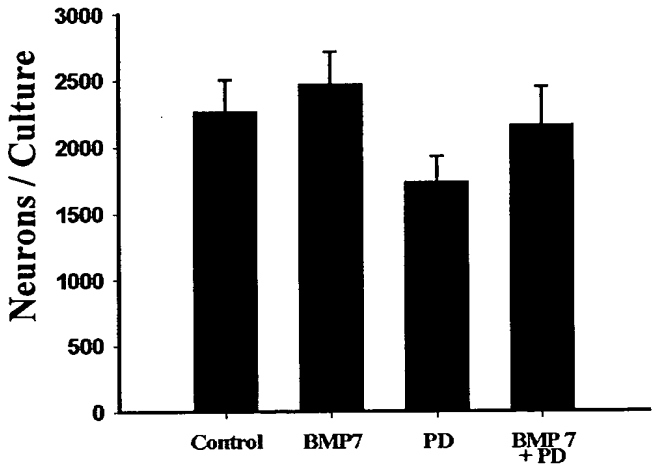
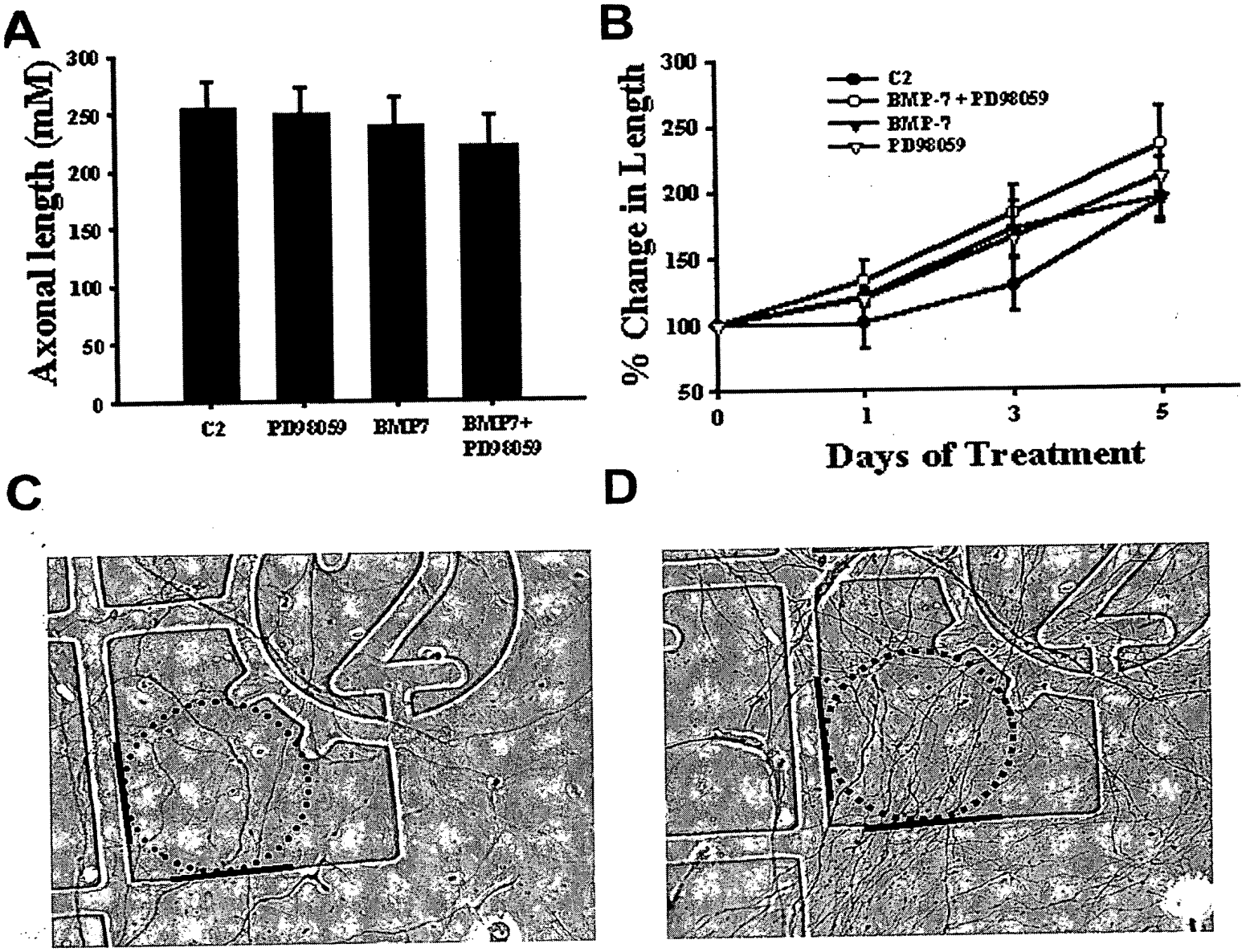

Neurons / Culture
3000
2500
2000
1500
1000
500
0
Control
BMP7
PD
BMP 7 + PD

Figure 7


A
Axonal length (mM)
300
250
200
150
100
50
0
C2
PD98059
BMP7
BMP7+
PD98059
B
% Change in Length
300
250
200
150
100
50
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1
3
5
Days of Treatment
C2
BMP-7 + PD98059
BMP-7
PD98059
C
D

Figure 8

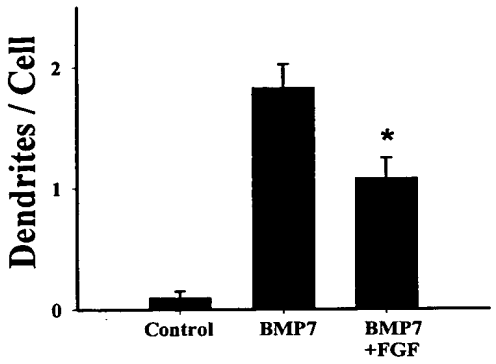

Figure 9

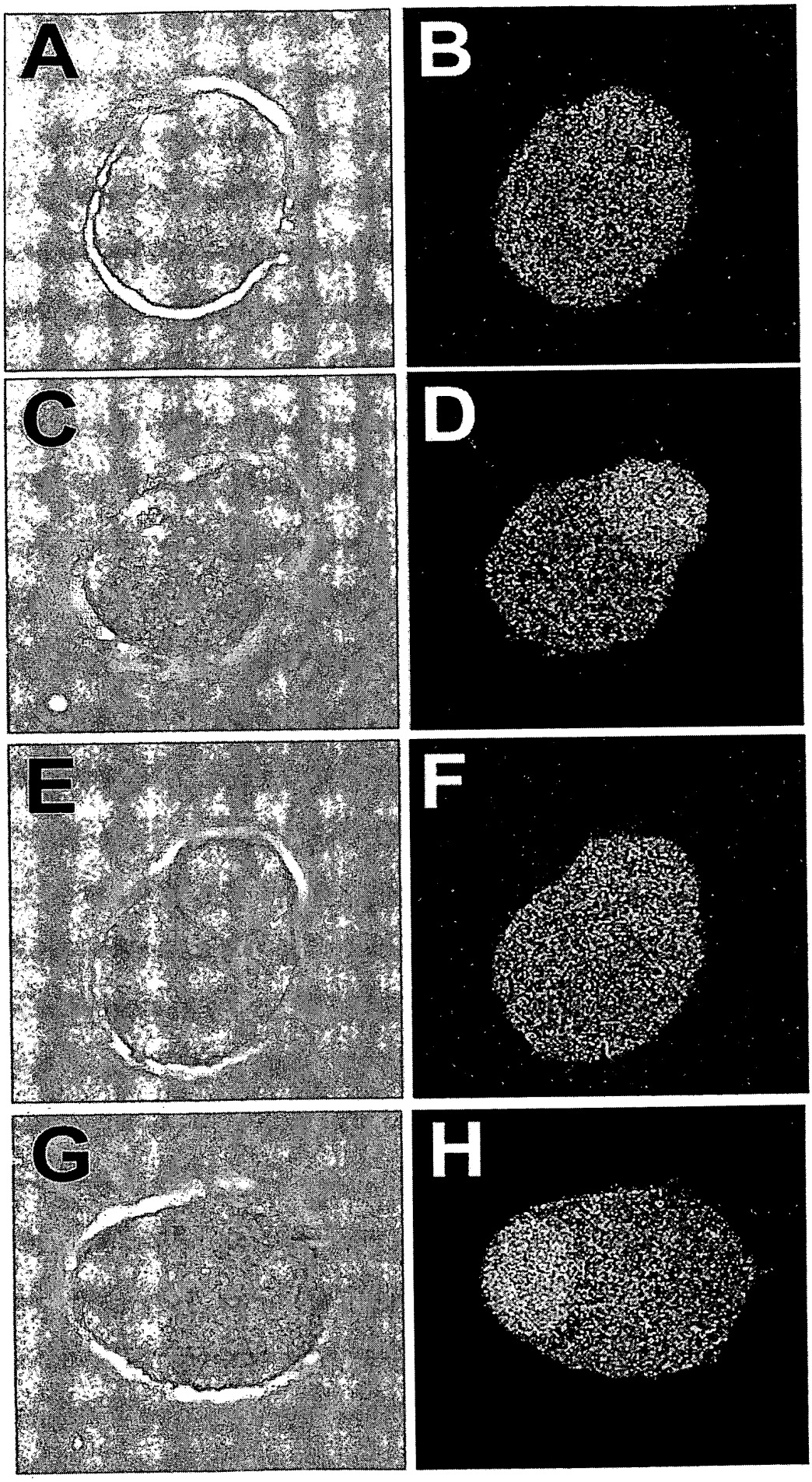

Figure 10

The Journal of Neuroscience, July 1988, 8(7): 2628–2634

Nerv Gr wth Fact r Enhances D ndritic Arb rization of Sympathetic Ganglion Cells in Dev l ping Mammal

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Recent observations have suggested that the dendritic arbors of sympathetic ganglion cells may be regulated by interactions with their peripheral targets (Voyvodic, 1987a; Yawo, 1987). In order to assess a potential mechanism for such interactions, I have investigated the effects of the target-derived trophic molecule for sympathetic ganglion cells on the development of dendrites in the rat superior cervical ganglion. Systemic treatment of neonatal animals with NGF f r 1 or 2 weeks results in a striking expansion of ganglion cell dendritic arbors, as revealed by intracellular staining with HRP. During this period, neurons in treated animals extend new primary dendrites, and the length and branching of existing dendrites are increased compared to age-matched controls. These results support the idea that targets may regulate ganglion cell arbors via elaboration of NGF, and suggest an explanation for the correlation between animal size nd dendritic complexity noted in several recent studies (Purves and Lichtman, 1985a; Snider, 1987; Voyvodic, 1987a).

Because the majority of synapses in the mammalian nervous system are found on dendrites, the regulation of dendritic arbors is of critical importance to neural organization. To some degree, dendritic arbors appear to be specified at early developmental stages by intrinsic genetic mechanisms (Banker and Cowan, 1979; Kriegstein and Dichter, 1983; Honig and Hume, 1986). However, arbors are also influenced by extrinsic factors, particularly neural connections (Grant 1968; Sumner and Watson, 1971; Benes et al., 1977; Parks, 1981; Deitch and Rubel, 1984). In the case of sympathetic ganglia, where both afferent and efferent projections are accessible and easily interrupted, the advent of intracellular staining has allowed a detailed examination of the influence of neural connections on dendritic arborization. Such studies have revealed that ganglion cell arbors are not appreciably influenced by afferent fibers, either during development or in maturity (McLachlan, 1974; Smolen and Beaston-Wimmer, 1986; Voyvodic, 1987a). On the other hand, arbors retract after axotomy and re-expand in association with reinnervation of targets (Purves, 1975; Yawo, 1987). These findings have suggested that dendritic arbors of sympathetic ganglion cells are primarily dependent on efferent connections, and thus may be regulated by interactions with their peripheral targets (Voyvodic, 1987a; Yawo, 1987). Further support for a target-derived influence on the arborization of sympathetic neurons has come from experiments showing a correlation between arbor complexity and target size during development (Voyvodic, 1987a), across species (Purves and Lichtman, 1985a; Purves et al., 1986; see also Snider, 1987), and after experimentally induced changes (Voyvodic, 1987b).

Neuronal interactions with targets are thought to be mediated by the uptake and retrograde transport of neurotrophic molecules (Harris, 1974; Purves and Lichtman, 1985b). That trophic molecules might affect dendritic arborization is suggested by the striking propensity of the prototype of these molecules, NGF, to promote neurite outgrowth from susceptible neurons *in vivo* and *in vitro* (for reviews, see Levi-Montalcini and Angeletti, 1968; Thoenen and Barde, 1980). Previous investigations have established that administration of NGF to developing mammals leads to profuse branching of the axons of sympathetic ganglion cells and expansion of their terminal arborizations in the periphery (Levi-Montalcini and Cohen, 1956; Olson, 1967; Levi-Montalcini and Angeletti, 1968). However, the influence of NGF on dendritic arbors has not been studied in a systematic manner. The purpose of the present study, therefore, was to ask whether administration of NGF affects dendritic arbors in a manner consistent with the idea that this molecule may regulate dendritic development in sympathetic ganglia.

Materials and Methods

NGF was administered daily to newborn Sprague-Dawley rats beginning on postnatal day 1 or 2. Purified β subunit of mouse NGF (kindly provided by E. M. Johnson, Jr. and P. Osborne) was dissolved in saline and injected subcutaneously in a dosage of 5 mg/kg. A similar dose is known to increase sympathetic ganglion cell size and enhance synthesis of transmitter enzymes (Thoenen et al., 1971). Animals were treated for either 7 or 14 d and compared with controls at these same ages.

For intracellular staining, the superior cervical ganglia were removed and pinned in a chamber superfused with an oxygenated mammalian saline. Neurons were impaled with triangular glass electrodes (Glass Co. of America) filled with a 4% solution of HRP (Sigma, type 6). The HRP was introduced into the cell by iontophoresis and the reaction product was visualized by the pyrocatechol–phenylenediamine method (Hanker et al., 1977; for details, see Purves and Hume, 1981; Foreband and Purves, 1984). Stained neurons were viewed at 300× in whole-mount preparations (Fig. 1) and traced with the aid of a camera lucida. Dendritic and axonal processes were distinguished using established criteria (Purves and Hume, 1981). Dendrites had numerous short processes arising from the main shaft and branched into secondary and tertiary segments relatively close to the cell soma (Fig. 1). The axon was readily identified as a smooth, thick process that usually could be followed for

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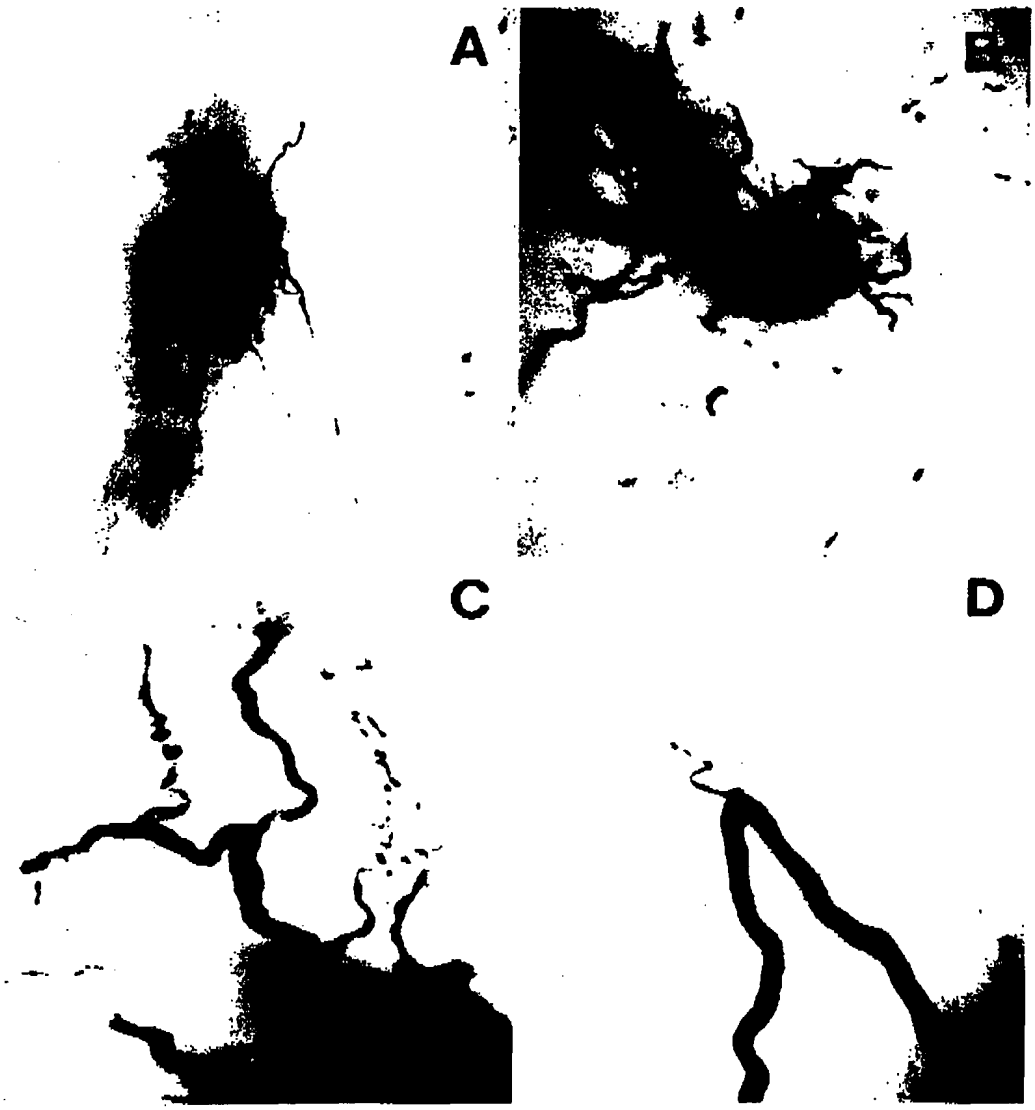


Figure 1. Photomicrographs of representative superior cervical ganglion cells stained with HRP and viewed in ganglion whole-mounts. *A*, Neuron from a control animal 2 weeks old. This cell had 8 primary dendrites and a total dendritic length of 919 μm. *B*, Neuron from an animal treated for 2 weeks with NGF. This cell had 12 primary dendrites and total dendritic length of 2328 μm. *C*, High-power view of representative dendritic processes. Note branching near the soma (*bottom right*) and irregularities along the shafts. *D*, High-power view of a representative axon in an animal treated with NGF. The axon surface is smooth and one fine branch is elaborated. Such axon branches are not seen in controls.

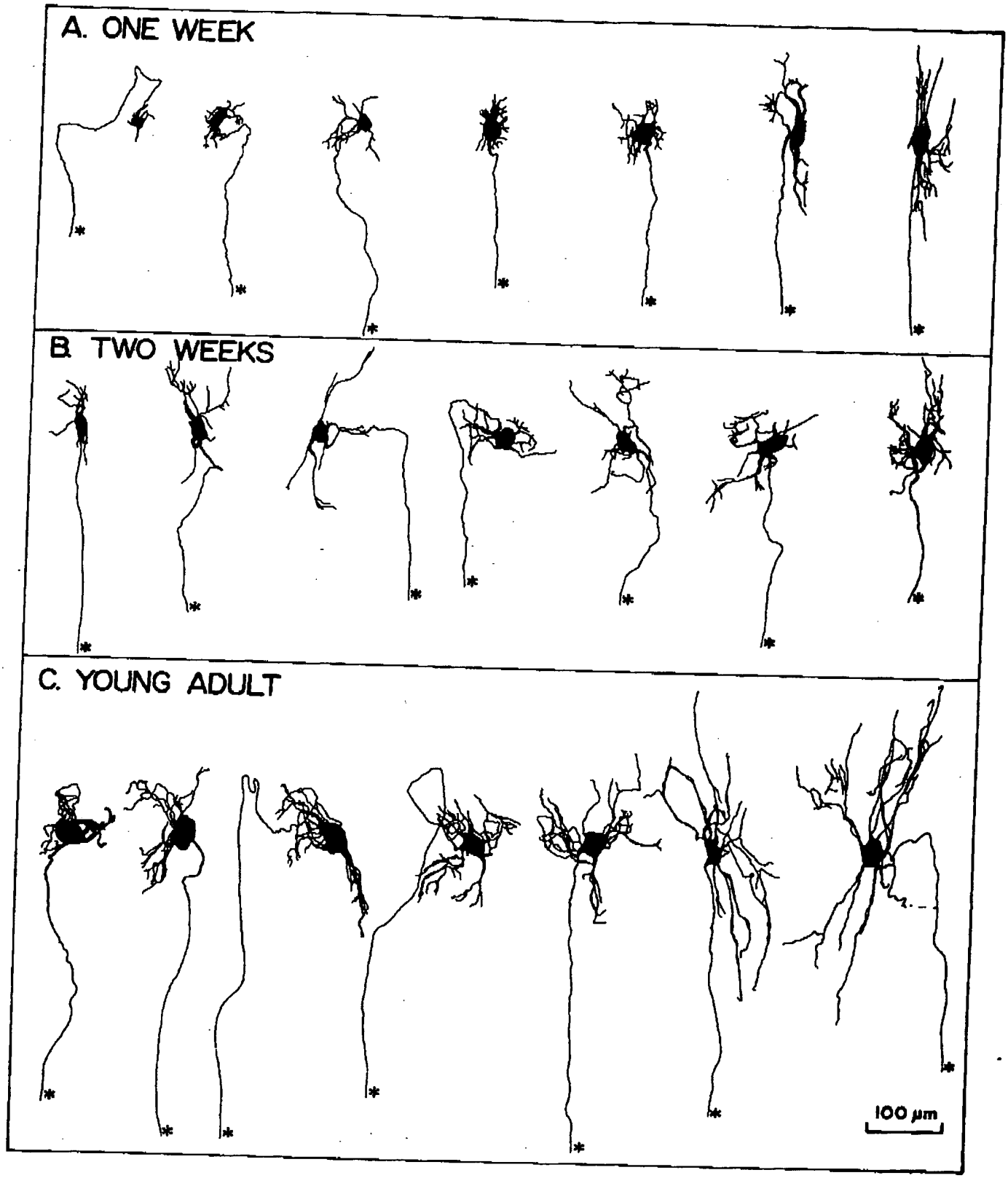
at least several hundred microns and frequently could be seen exiting the ganglion via a postganglionic nerve. In control ganglia, neurons invariably possessed a single axon, whereas a few cells in the NGF groups had 2. Also in the NGF group, axons frequently (approximately 50%) elaborated branches within the ganglion, a feature not seen in controls (Fig. 1). Occasional processes in treated animals could not be clearly categorized and were not included in the quantitative analysis.

The arbor of each neuron was assessed by 4 measures of dendritic complexity. The number of primary dendrites was determined by viewing the cells at 480× in multiple focal planes. A primary dendrite was defined as any process extending from the soma a distance greater than the cell diameter. Total dendritic lengths were measured from the camera lucida tracings with the aid of a digitizing tablet and a general purpose program for neural imaging (Voyvodic, 1986). The radius of a circle incorporating the entire arbor was measured as an indicator of process length. Finally, the extent of branching was determined by counting the number of branches crossing a 50% circle (Scholl, 1953).

All well-stained neurons were analyzed. At 1 week, 47 neurons from 6 ganglia in NGF-treated animals were compared with 39 neurons in 10 ganglia from controls. Morphologic data on some cells in the 1 week control group are from a previous study (Snider, 1986). At 2 weeks, 43 cells in 6 ganglia from NGF-treated animals were compared with 36 cells in 8 ganglia from controls.

Results

The dendritic arbors of sympathetic ganglion cells are quite rudimentary in neonatal animals (Fig. 2; see also Snider, 1986; Voyvodic, 1987a). At 1 week of age, the mean total dendritic


A. ONE WEEK
B. TWO WEEKS
C. YOUNG ADULT
100 µm

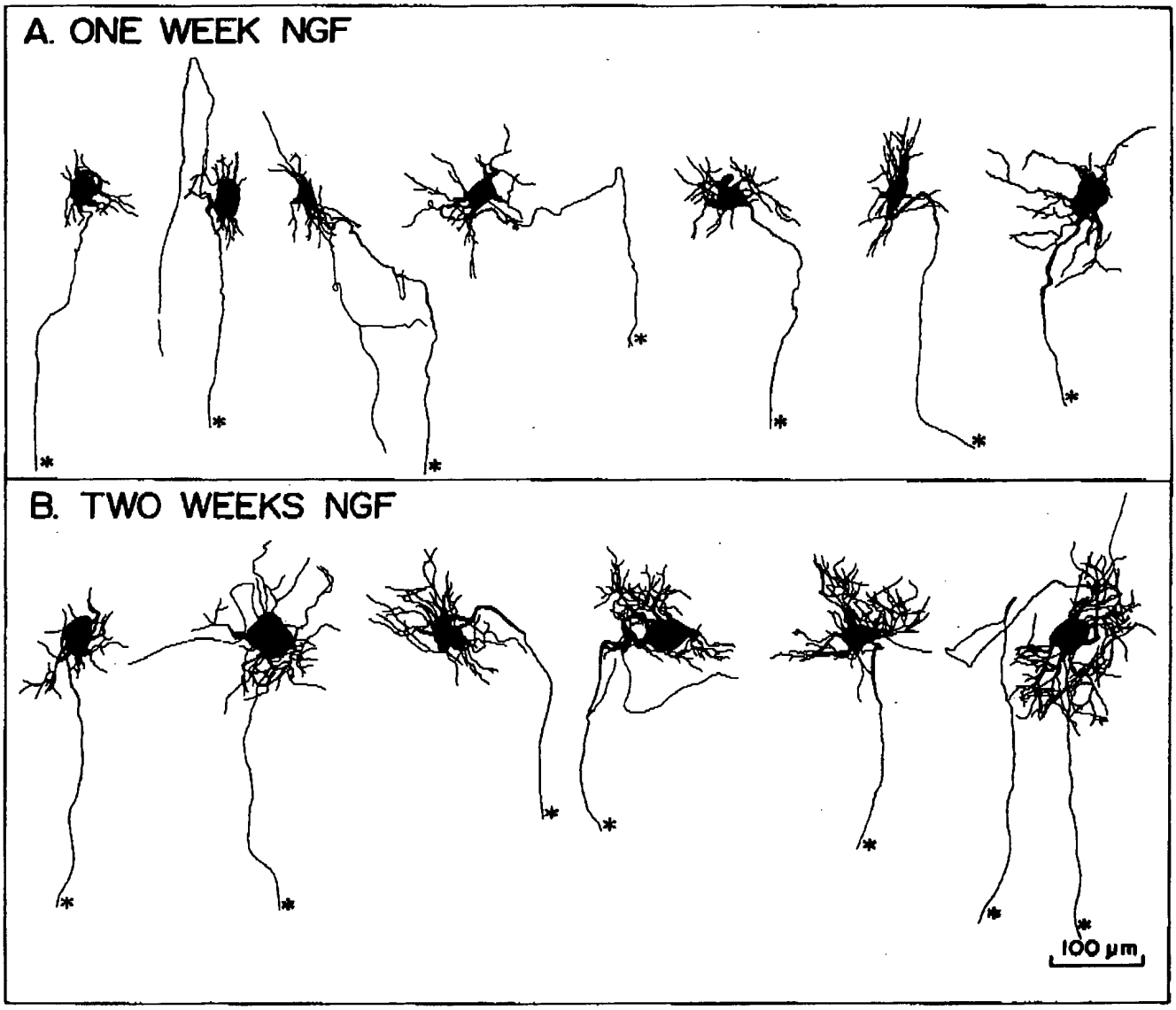


Figure 3. Effects of daily administration of NGF on dendritic arbors of sympathetic ganglion cells. The scale is the same as Figure 2. *A*, One week of treatment. *B*, Two weeks of treatment. Neurons were arranged in order of increasing total dendritic length, and every seventh neuron selected. Neurons 2 and 3 in *A* and 4 and 6 in *B* exhibit axonal branches. Neuron 2 in *B* has a process (exiting to left) that could not be clearly classified. Such processes were not included in the calculation of total dendritic length.

length in the superior cervical ganglion is only about one-third that of young adults (see Table 1). Ganglion cell arbors expand rapidly in the first few weeks after birth. Thus, almost 200 µm of dendritic length is added between postnatal weeks 1 and 2 (Table 1). Measures of process length and branching increase in parallel with increases in total dendritic length during this period. However, one important component of arbor geometry, the number of primary dendrites, does not change during normal postnatal development (Fig. 2; Table 1). This finding raises the possibility that various aspects of dendritic growth may be differentially regulated (see below; see also Voyvodic, 1987a).

Administration of NGF had a pronounced effect on dendritic

←

Figure 2. Normal development of sympathetic ganglion cells over the first 8 weeks of life. For *A–C*, camera lucida drawings were arranged in order of increasing total dendritic length and every sixth (*A*) or fifth (*B*) neuron was selected. (*C* is from Snider, 1986, and is shown for purposes of comparison with neurons from the animals treated with NGF for 2 weeks.)

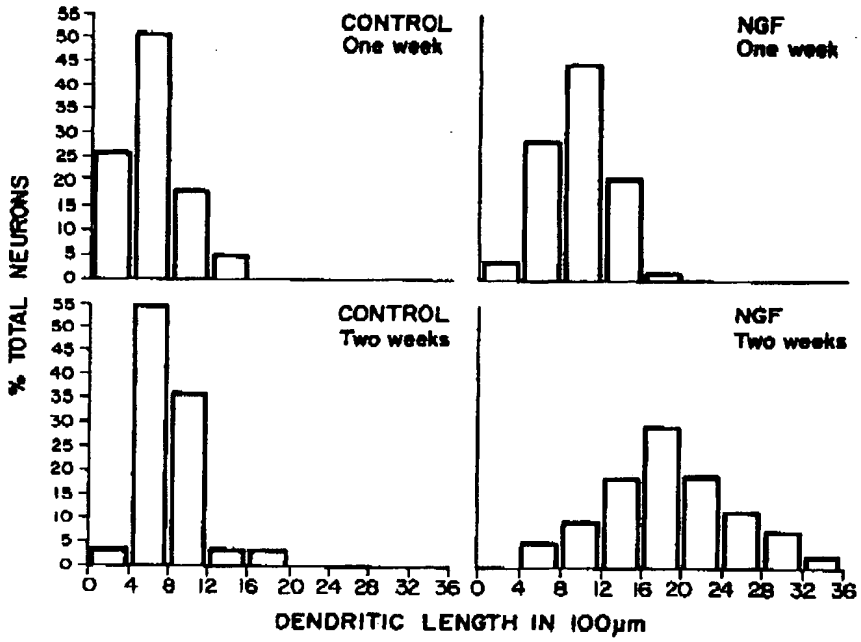


Figure 4. Histograms of dendritic lengths of control (*left*) and NGF-treated (*right*) animals. There is considerable variability in both the treatment and control groups.

arborization (Fig. 1; cf. Figs. 2, 3). After just 1 week of treatment, there were highly significant increases in process length, extent of branching, and total dendritic length (Table 1, Fig. 4). After 2 weeks, the mean total dendritic length of ganglion cells in treated animals was more than twice that of control neurons (1885 μm compared to 779 μm) and similar to neurons in young adults 8 weeks of age. Since rat superior cervical ganglion cells have a mean total dendritic length of 287 μm on the day of birth (Yvodic, 1987a), these data indicate that neurons in NGF-treated animals extend dendrites at about 3 times the rate of controls over the 2 week period. Interestingly, neurons appeared to be at least as responsive during the second week of treatment as during the first.

In addition to its effects on other measures of complexity, treatment with NGF led to a large increase in the number of primary dendrites (cf. Figs. 2, 3; Table 1). In controls, ganglion cells had a mean of about 7 primary dendrites at 1 and 2 weeks, with 13 being the maximum elaborated by any neuron. In contrast, treated neurons at 1 week had a mean of almost 9 primary dendrites. After 2 weeks of NGF administration, neurons had a mean of 13 primary dendrites, with some neurons possessing more than 20. This increase in the number of primary dendrites caused ganglion cells in treated animals to look quite different from cells of equivalent total dendritic length in control animals 8 weeks of age (cf. Figs. 2, 3).

Finally, as has been apparent from the earliest days of work with this molecule, NGF has a remarkable effect on the size of sympathetic ganglion cell bodies (cf. Figs. 2, 3; Table 1). After 1 week of treatment, soma diameter was increased by almost 25% compared to controls. After 2 weeks, soma diameter in treated animals was 30% more than in 2-week-old controls and 20% more than in young adult animals.

Discussion

The results described here demonstrate that exogenous NGF enhances the dendritic arborization of developing sympathetic ganglion cells. Neurons in treated animals had more primary processes than controls, and the length and ramification of dendrites were increased substantially. Significant effects were noted after just 1 week of treatment. After 2 weeks, the overall com-

Table 1. Morphological characteristics of superior cervical ganglion cells in control and NGF-treated animals

	Soma diameter (μm)	Primary dendrites		Total dendritic length (μm)	Maximum extent of dendritic arbor (μm)	Branches crossing 50% circle
		Mean	Range			
1 week control	26.0 ± 4.9	7.0 ± 2.7	1–13	589 ± 313	78 ± 32	6.4 ± 2.7
2 week control	29.2 ± 6.0	6.9 ± 2.1	4–13	779 ± 288	99 ± 31	8.0 ± 2.8
Young adult	31.3 ± 5.7	7.7 ± 2.6	5–15	1655 ± 635	125 ± 43	9.9 ± 4.0
1 week NGF	32.1 ± 4.0	8.7 ± 3.4	4–15	948 ± 307	91 ± 21	11.1 ± 4.0
2 week NGF	38.3 ± 7.0	13.3 ± 4.1	5–23	1885 ± 652	118 ± 32	19.6 ± 9.2

All values except range of primary dendrites are given as means ± SDs. Results were analyzed using Student's *t* test. The differences in number of primary dendrites and maximum extent of dendritic arbor between the 1 week control and NGF groups were significant at $p < 0.01$. The difference in maximum extent of the arbor between the 2 week control and NGF groups was significant at $p < 0.005$. All other differences between control and NGF groups were highly significant (at least $p < 0.001$). Values for young adult are from Snider (1986).

plexity of treated neurons was more than twice that of controls and similar to the total extent of ganglion cell dendrites in animals 8 weeks of age.

In comparing the dendritic growth induced by NGF to the growth of arbors that occurs during normal development, important similarities and differences are apparent. Normally, the number of primary dendrites is set by the first postnatal week, and arborization proceeds by increases in length and branching of existing processes (see also Snider, 1986; Voyvodic, 1987a). A posssible explanation for this pattern of arborization is that sympathetic neurons either cannot respond to target-derived factors by generating new primary processes or respond in this way only during embryonic life. My results make this idea unlikely. Exogenous NGF promotes the growth and branching of existing dendrites, as occurs during normal postnatal development. In addition, neurons extend new primary processes under the influence of NGF even in the postnatal period. This ability to change normal geometry also indicates that NGF does more than simply accelerate a preexisting developmental program.

My results imply that arbors of sympathetic ganglion cells may be influenced by target-derived NGF during normal development. Although it is not known how NGF acts after systemic administration, it is likely that it mimics the effects of target-derived factor. Previous investigations have established that exogenous NGF can prevent naturally occurring cell death (like a supernumerary target) and maintain ganglion cells after separation from their targets by axotomy (Hendry and Campbell, 1976; Hamburger and Yip, 1984; Yip et al., 1984). Systemic NGF may exert additional effects, however, by local action on neurites in the ganglion (see Campenot, 1977, 1982a, b).

The demonstration that dendritic arbors of autonomic ganglion cells are influenced by trophic molecules may provide an explanation for correlations between arbor complexity and target size noted in recent investigations. The dendritic complexity of rat superior cervical ganglion cells increases in parallel with increasing body weight (and target size) for much of the life of the animal—far beyond the time normally considered to be the period of development (Voyvodic, 1987a). Furthermore, experimental increases and decreases in the amount of target tissue innervated by individual ganglion cells during development lead to corresponding changes in their dendritic complexity (Voyvodic, 1987b). Finally, the dendritic arbors of homologous autonomic ganglion cells in closely related species of differing size exhibit systematic differences in length and complexity that are correlated with body weight (Purves and Lichtman, 1985a; Purves et al., 1986; Snider, 1987). As the amount of trophic factor produced by a target organ presumably bears some relationship to its size, the ability of NGF to influence arbors provides a plausible link between the dendritic complexity of innervating neurons and the size of peripheral targets.

These results may also have an important implication for the organization of peripheral motor systems. Experiments that have examined the morphology and innervation of autonomic ganglion cells have shown that the number of innervating axons (preganglionic convergence) is closely matched to dendritic complexity in maturity (Purves and Hume, 1981; Purves and Lichtman, 1985a; Snider, 1987). Factors that regulate the morphology of ganglion cells will therefore have a significant influence on their pattern of innervation. Although the effects of NGF on preganglionic convergence have not yet been studied, on the basis of the present results, NGF might be expected to influence the number of inputs to sympathetic neurons. In support of this suggestion, NGF administered to neonatal rats results in an increase in the number of axons in the cervical sympathetic trunk and in the density of synaptic boutons in the superior cervical ganglion (Schäfer et al., 1983). Thus, both the morphology and innervation of sympathetic ganglion cells may be matched to the size of their peripheral targets via the uptake of NGF.

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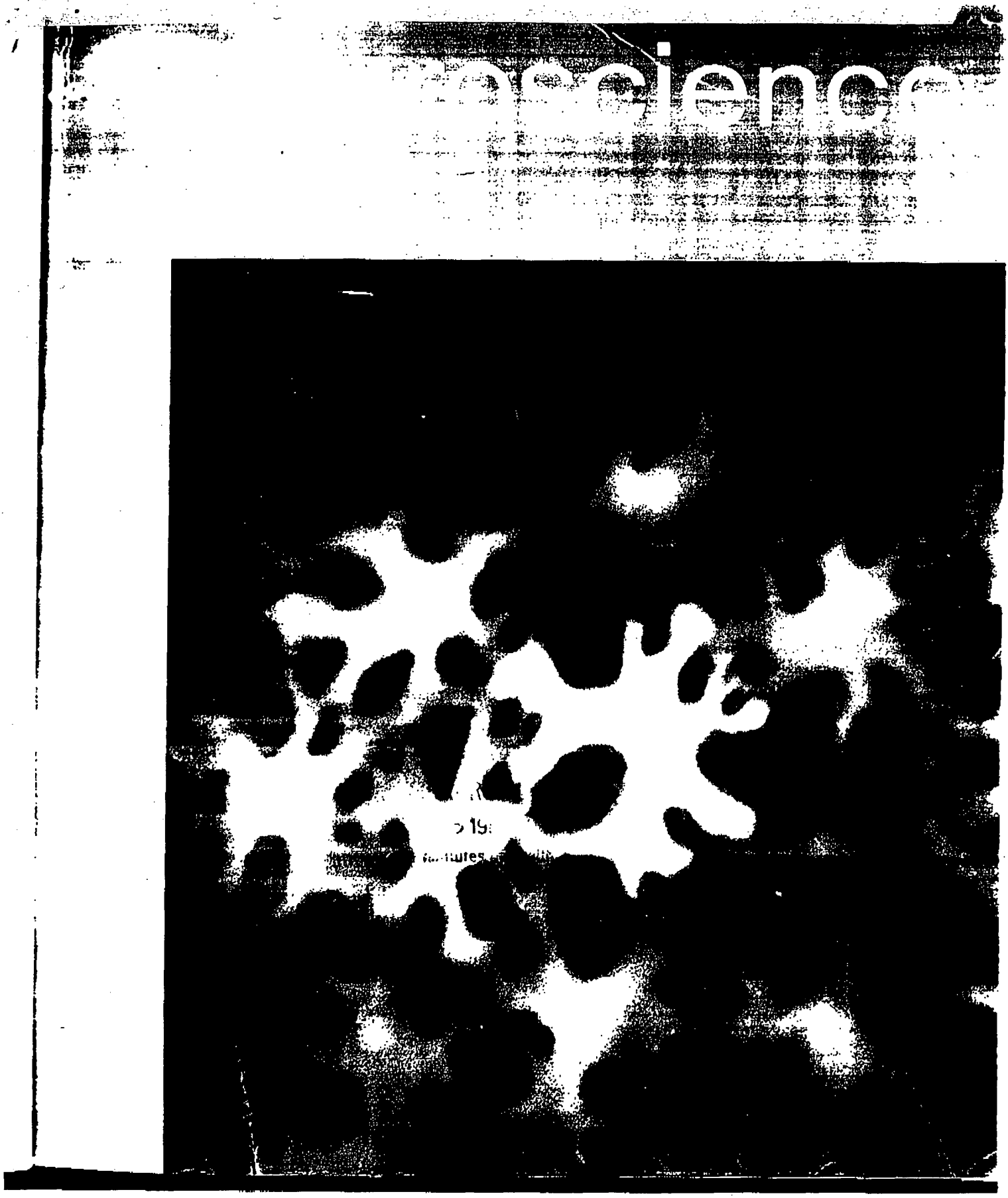

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Osteogenic Protein-1 Induces Dendritic Growth in Rat Sympath tic Neurons

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Summary

Sympathetic neurons from perinatal rat pups extend only a single axon when maintained in culture in the absence of glia and serum. Exposure to recombinant osteogenic protein-1 (OP-1) selectively induces the formation of dendrites that correctly segregate and modify cytoskeletal and membrane proteins and form synaptic contacts of appropriate polarity. OP-1 requires nerve growth factor (NGF) as a cofactor, and, in the presence of optimal concentrations of NGF, OP-1-induced dendritic growth from cultured perinatal neurons is comparable to that observed in situ. Sympathetic neuroblasts that had not formed dendrites in situ also responded to OP-1 in culture, indicating that OP-1 can cause de novo formation as well as regeneration of dendrites. These data imply that specific signals can regulate the development of neuronal shape and polarity.

Introduction

Dendrites are the primary site of synapse formation in the vertebrate nervous system, and neurons that lack dendrites typically receive fewer synaptic inputs than cells with complex dendritic arbors (Purves, 1988). Therefore, to understand how the number of afferent synapses is determined, it is necessary to identify the molecules that regulate the growth of dendrites.

Dendritic growth can be considered to occur in two phases: initial extension followed by elongation and ramification. Many molecules, including neurotransmitters, hormones, and neurotrophic factors, have been shown to modulate the expansion of the dendritic arbor (Kelly, 1988; Mattson, 1988; Snider, 1988). Less is known about the factors that cause a neuron initially to form dendrites. Hippocampal neurons generate substantial dendritic arbors when they are cultured in the absence of their targets, of n rmal aff rent input, and of contact with either glia or other hippocampal neurons (Dotti et al., 1988). These data suggest that, in certain classes of neurons, initial dendritic sprouting occurs as part of an intrinsic dev l pmental program and that execution of this program is relatively independent of trophic interactions. However, the initial stages of dendritic growth appear to be regulated differently in other classes of neurons. For example, rat sympathetic neurons fail to form dendrites and extend only axons when they are cultured in the absence of nonneuronal cells. In contrast, coculture with Schwann cells or astrocytes causes these neurons also to form dendritic processes and eventually to generate an arbor that is comparable in size to that observed in situ (Tropea et al., 1988; Johnson et al., 1989). Since this change in cell shape is not observed with fibroblasts or heart cells, it would appear that specific trophic interactions are required to allow sympathetic neurons to form dendrites.

Trophic regulation of the initial stages of dendritic growth has also been observed in cultures of mesencephalic, striatal, cerebral cortical, and preganglionic sympathetic neurons (Denis-Donini et al., 1984; Chamak et al., 1987; Clendening and Hume, 1990; Le Roux and Reh, 1994), suggesting that it may be a fairly common mechanism for determining neuronal shape. However, little is known about the molecules that cause neurons to form dendrites. Indeed, the only trophic factor that has been clearly implicated in the regulation of the initial stages of dendritic growth is nerve growth factor (NGF). This growth factor causes a subpopulation of nodose neurons to form dendrites in culture (de Koninck et al., 1993). Because these neurons lack dendrites in situ, the physiological significance of this effect is unclear. The observation is, however, consistent with the idea that there are trophic interactions that cause cells to form dendrites. NGF also enhances dendritic growth in sympathetic neurons when injected in situ (Snider, 1988). However, by itself, NGF does not support dendritic growth in cultures of sympathetic neurons (Bruckenstein and Higgins, 1988). It would therefore appear that there must be other molecules that regulate the morphological development of neurons.

OP-1, which is also known as bone morphogenetic protein-7 (BMP-7), is a member of the BMP/OP subfamily of the transforming growth factor β (TGFβ) superfamily (Sampath et al., 1992; Sampath and Rueger, 1994). Other members of this family include OP-2 (Ozkaynak et al., 1992) and BMP-2 through BMP-6 (Wozney, 1993). Many of these proteins were discovered using assays that measured de novo bone growth in vivo. However, there are indications that members of this subfamily also play a role in neural development. For example, mRNAs for OP-1, BMP-2, BMP-3, BMP-4, and BMP-5 have been detected in at least one region of the brain (Jones et al., 1991; Ozkaynak et al., 1992; Wozney, 1993), and immunocytochemical studies indicate that BMP-6 is prominently expressed in most structures of the embryonic peripheral nervous system (Wall et al., 1993). Other studies indicate that OP-1 increases expr ssion of th adr n rgic phenotype in neural crest cells (Varley et al., 1995) and regulates expression of L1 and neural cell adhesion molecule in a neural cell line (Perides t al., 1993), whereas BMP-2 and

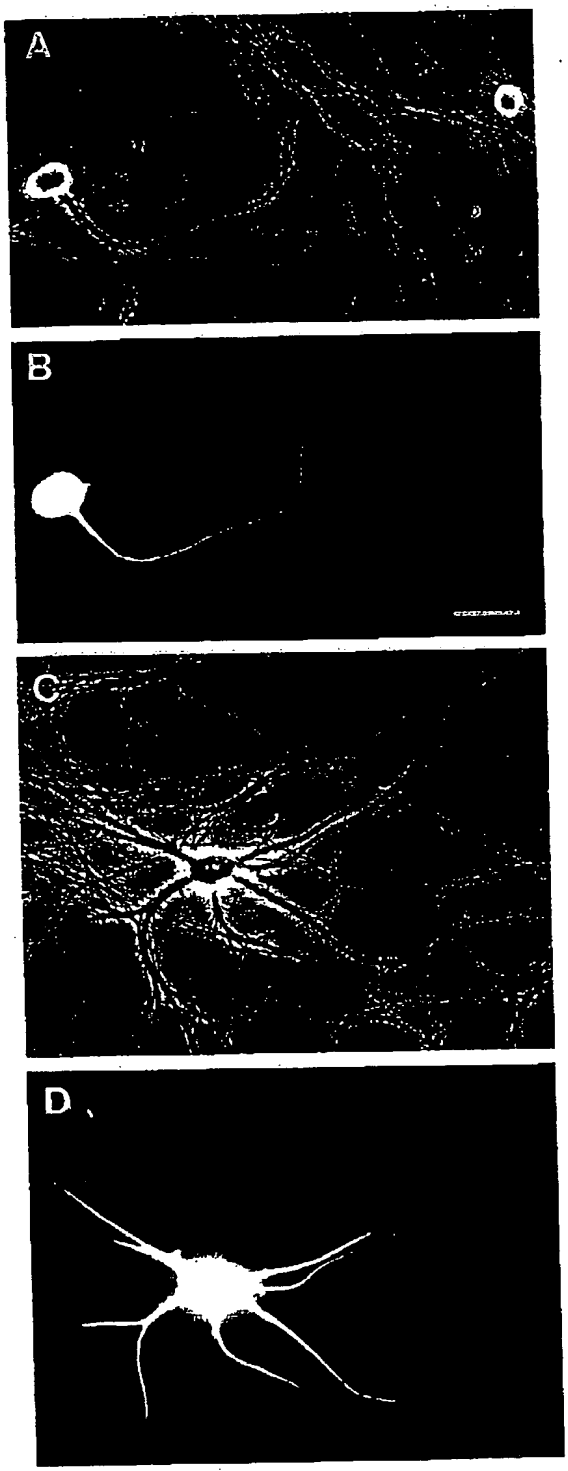

Figure 1. Effects of OP-1 on the Morphological Development of Sympathetic Neurons

Phase-contrast (A and C) and fluorescence (B and D) micrographs of neurons injected with Lucifer yellow during the third week in vitro. Neurons in control cultures (A and B) typically had only 1 process, a long axon. Neurons exposed to OP-1 (C and D) were multipolar, having several tapered dendrites and 1 axon. There was a tendency for the axons of OP-1-treated neurons to form small fascicles on polylysine. However, this was not observed on other substrates, such as laminin, which also supported OP-1-induced dendritic growth, suggesting that it is an epiphenomenon unrelated to the growth of dendrites. Bar, 50 μm.

BMP-6 affect the neurotransmitter phenotype of sympathetic neurons (Fann and Patterson, 1994). Moreover, a recently discovered member of the BMP/OP subfamily, dorsalin, was cloned from a spinal cord cDNA library and subsequently found to affect the differentiation of both neural crest cells and motor neurons (Basler et al., 1993).

This study examines the effects of OP-1 on perinatal rat sympathetic neurons. Our data indicate that OP-1 specifically induces dendritic growth in these cells, that it requires NGF as a cofactor, and that simultaneous exposure to both of these trophic factors causes the rate of expansion of the dendritic arbor in vitro to exceed that normally observed in situ. These observations suggest that, in addition to known trophic interactions that affect the survival and neurotransmitter phenotype of sympathetic neurons (Thoenen and Barde, 1980; Patterson and Landis, 1992), there may be others that specifically regulate their morphological development.

Results

OP-1 Induces Dendritic Growth in Sympathetic Neurons

Sympathetic neurons were dissociated from the superior cervical ganglia of perinatal rats. They were then plated onto polylysine-coated coverslips and maintained in a serum-free medium that contained NGF. Nonneuronal cells were eliminated by treatment with an antimitotic agent on days 2 and 3. One to 2 days were then allowed for recovery before beginning experimental treatments on day 5 or 6. Cellular morphology was initially assessed by intracellular dye injection.

Under control conditions, sympathetic neurons typically extend a single process during the first 24–48 hr in vitro. Previous studies have shown that this process has the cytoskeletal and ultrastructural characteristics of an axon (Tropea et al., 1988; Lein and Higgins, 1989). The axon continues to elongate during the next few weeks and generates an elaborate plexus (Figure 1). However, the basic morphology of the cells remains essentially unchanged (Figure 2), with 80% of the neurons still being unipolar after 1 month. Most of the remaining population had either 2 axons (13% of the cells) or an axon and a short dendrite (7%). Thus, the mean number of processes at this time was 1.13 ± 0.06 (n = 30) axons/cell and 0.07 ± 0.04 dendrites/cell.

Exposure to recombinant human OP-1 caused sympathetic neurons to form additional processes (see Figure 1). The response was relatively slow, with only 42% of the cells forming a second process within 24 hr (see Figure 2). However, virtually all cells responded to maximally effective concentrations within 3 days. The processes that

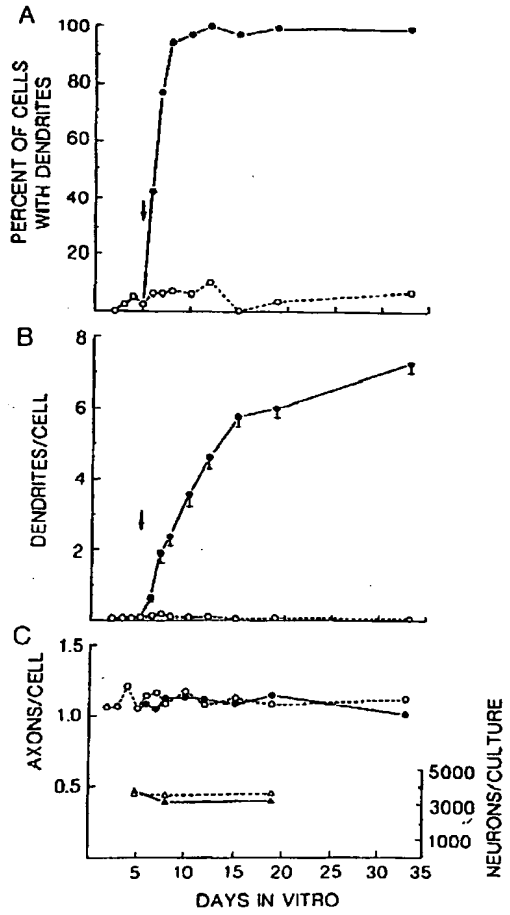

Figure 2. Time Course of the Response to OP-1

Sympathetic neurons were maintained in serum-free medium for 4 days. Beginning on day 5 (arrows), the medium of some cultures was continuously supplemented with OP-1 (100 ng/ml). Intracellular dye injections ($n > 30$ per point) were performed at various times to determine: (A) the percentage of cells with dendrites (open symbols, control; closed symbols, OP-1); (B) the number of dendrites per cell; and (C) the number of axons per cell (circles). Cell number (triangles in [C]) remained constant during the experiment. Bars (B) represent the SEM; where bars are not shown, SEM was smaller than the size of the symbol.

formed in the presence of OP-1 had the appearance of dendrites in that they were broad-based (up to 6 μm in diameter), exhibited a distinct taper, and branched in a "Y"-shaped pattern, with daughter processes being significantly smaller than the parent process (see Figure 1). Dendrites were much thicker than axons, and unlike axons, they ended locally, usually extending less than 300 μm from the soma. The number of dendrites per cell continued to increase during a 4 week exposure to OP-1, with most of the change occurring during the first 10 days of treatment (see Figure 2). After 4 weeks, OP-1-treated neurons had a mean of 7.3 ± 0.3 ($n = 30$) dendrites/cell, representing an increase of ~100-fold over control cells.

Exposure to OP-1 did not cause an increase in the number of axons per cell when it was tested in the delayed introduction paradigm used in the experiment shown in Figure 2. We also examined the effects of OP-1 on initial axon growth during the first 48 hr in culture. Neither the rate at which axons were initially extended nor the mean number of axons per cell was affected (data not shown). It therefore appears that OP-1 affects sympathetic neurons in a process-specific manner.

Cell number also remained constant during the exposure to OP-1 (see Figure 2), indicating that it was not acting by enhancing the survival of a subpopulation of neurons. However, the somata of neurons treated with OP-1 were larger than those of control cells (see Figure 1). The significance of this change is unclear, but since somatic hypertrophy is also associated with sympathetic dendritic growth in situ (Voyvodic, 1987), it could simply reflect the added synthetic capacity needed to sustain the growth of additional processes.

Properties of Dendrites Formed in the Presence of OP-1

When cultures exposed to OP-1 were immunostained with a monoclonal antibody (MAb) to microtubule-associated protein-2 (MAP2), immunoreactivity was observed in the somata and dendritic processes, but not in the thinner axons (Figure 3). Moreover, the mean number and average length of the MAP2-positive processes corresponded closely (within 10%) to the values obtained from dye injections, suggesting that all dendrites were stained in their entirety. A similar staining pattern was observed with MAbs to nonphosphorylated neurofilaments (Figure 3) and to the transferrin receptor (data not shown). In contrast, MAbs to tau (Figure 3), synaptophysin, or phosphorylated forms of the H or the M and H neurofilament subunits (data not shown) selectively stained the thin axons, with little or no immunoreactivity observed in the dendrites.

When cultures treated with OP-1 were examined in the electron microscope, tapered dendrites were frequently observed emanating from neural somata. The dendritic cytoplasm was continuous with that of the soma, and proximal dendrites contained both polyribosomes and occasional short segments of rough endoplasmic reticulum. Polyribosomes were also observed in more distal dendrites at distances greater than 100 μm from the soma but were not observed in axons. In contrast, synaptic vesicles were seen only in axons. Dendrites were typically smoothly contoured and lacked the spinelike protrusions observed on some sympathetic neurons in situ (Matthews, 1983). However, the synaptic contacts that were found along dendrites had an appropriate polarity; both presynaptic axonal accumulations of vesicles and postsynaptic densities were observed.

Concentration-Effect Relationship and Comparison of OP-1 with Other Growth Factors

The effects of OP-1 were concentration dependent (Figure 4A). Maximal dendritic growth was obtained with concentrations between 30 and 100 ng/ml, and half-maximal effects were observed at ~2 ng/ml. However, significant changes in dendritic growth could be detected with concentrations as low as 300 pg/ml. These data suggested

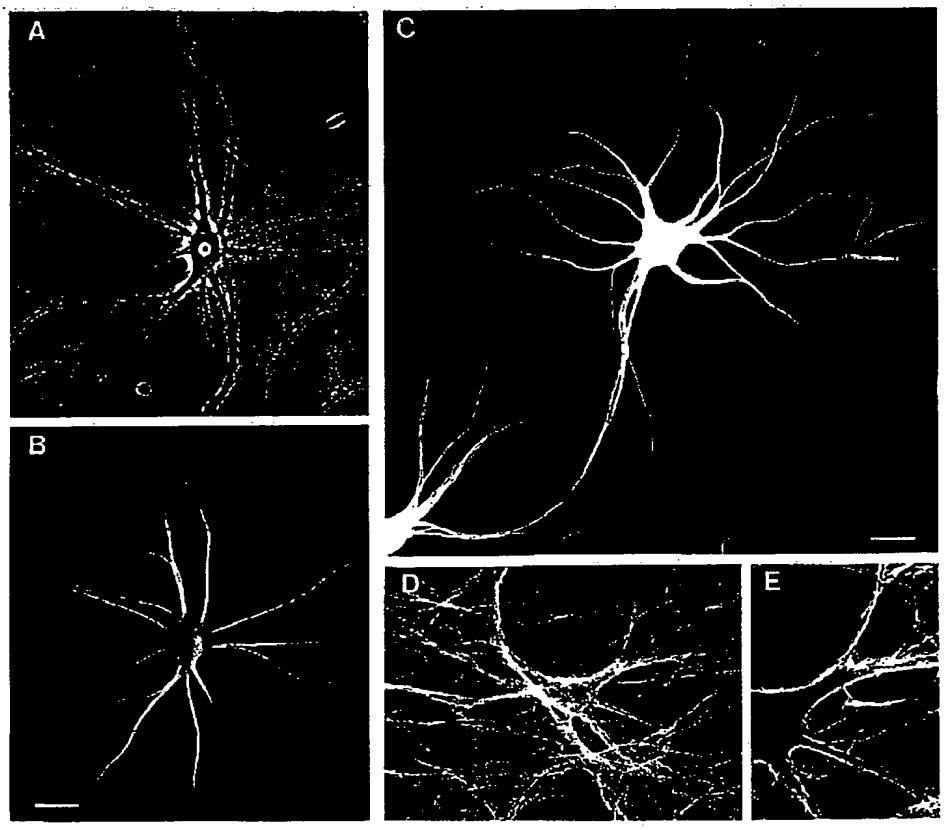

Figure 3. Cytochemical Characteristics of Processes Formed in the Presence of OP-1

Phase-contrast (A) and fluorescence (B) micrographs of a neuron that was exposed to OP-1 for 18 days before being immunostained with a MAb (AP13) to MAP2. Immunoreactivity was observed primarily in the soma and dendrites, although there was occasional labeling of an axon (B, lower right). A similar staining pattern (C) was observed in cultures reacted with a MAb (SMI-32) to nonphosphorylated forms of the M and H neurofilament subunits (21 day exposure to OP-1). In contrast, prominent axonal labeling was observed in cultures reacted with a MAb to tau protein (D); an enlargement is shown in (E). Label was also observed in the region of the cell body and dendrites, but confocal microscopy (1 μm optical sections) revealed that it was primarily associated with axons coursing over the surface of these structures (E). Bars, 50 μm (B and C).

a specific effect of the recombinant protein upon neurons, and this was confirmed by antibody blocking experiments. MAbs 1B12 and 12G3 have previously been shown (Vukicevic et al., 1994) to react with OP-1 but not with closely related molecules, such as BMP-2 and BMP-4. When added to the medium at a concentration of 5 μg/ml, MAbs 1B12 and 12G3 inhibited OP-1-induced (2 ng/ml) dendritic growth by 71% and 100%, respectively.

Since OP-1 belongs to the TGFβ superfamily, its actions were compared with those of other family members. Dendritic growth was not observed in the presence of TGFβ1, TGFβ2, TGFβ3, activin A, inhibin, or glia-derived neurotrophic factor (all tested at 100 ng/ml). Negative results were also obtained with similar concentrations of members of the neurotrophin (NT) family (NT3, NT4, and brain-derived neurotrophic factor), basic fibroblast growth factor, pidermal growth factor, ciliary neurotrophic factor, leukemia inhibitory factor, hepatocyte growth factor, granulocyt /macrophage colony-stimulating factor, Γ interferon, platelet-derived growth factor, TGFα, vascular endothelial growth factor, and interleukin-1β, -2, -3, -4, -6, -7, and -8. These data suggest that the dendrite-promoting effect of OP-1 is a specific response that is not observed with most of the other growth factors known to affect neurons.

Effects of NGF on OP-1-Induced Dendritic Growth

NGF regulates the growth of sympathetic dendrites in situ (Snider, 1988), but dendritic growth was not observed in our control medium, which contained high levels of NGF. We therefore considered the hypothesis that NGF functions as a modulator rather than an inducer of dendritic growth. Neurons were exposed to a maximally effective concentration of OP-1 while the amount of NGF was varied. As the concentration of NGF was decreased from 100 to 0.3 ng/ml (Figure 4B), there was a concentration-dependent decrease in the number of dendrites per cell and in the percentage of cells with dendrites. Cell number decreased over this same concentration range, and the half-maximal concentration for cell survival differed less

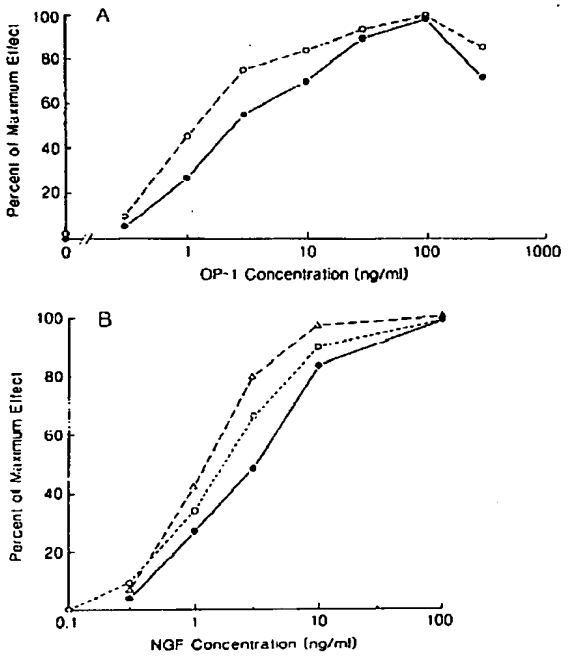

Figure 4. Effects of Varying Concentrations of OP-1 and NGF on Dendritic Growth

(A) Sympathetic neurons were exposed to varying concentrations of OP-1 for 3 days and then immunostained with a dendrite-specific antibody (SMI-32) to determine the percentage of cells with dendrites (open circles) and the number of dendrites per cell (closed circles). Data are expressed as a percentage of the maximum value obtained in the presence of 100 ng/ml OP-1 (100% of the cells with dendrites; 3.96 ± 0.32 dendrites/cell).

(B) Neurons were grown in media containing varying concentrations of NGF. Beginning on day 5, they were treated with a maximally effective concentration (100 ng/ml) of OP-1. One week later, they were immunostained to determine the percentage of cells with dendrites (triangles; maximum value, 97%), the number of dendrites per cell (closed circles; maximum value, 4.7 ± 0.3), and the number of neurons surviving per culture (open circles; maximum value, 2808 ± 267).

than 2-fold from that for either the percentage of cells with dendrites or the number of dendrites. These data suggest that NGF is a necessary cofactor for OP-1-induced dendritic growth and that the amount of dendritic growth that occurs is critically dependent on the trophic state of the cell.

A separate experiment directly examined the acute effects of OP-1 on neuronal survival in the presence and absence of NGF. In control medium containing NGF (100 ng/ml), 63% of the neurons plated survived for 48 hr under our low density culture conditions (9455 ± 445 neurons/22 mm well). A similar number (9641 ± 686 neurons/well) survived in the presence of both NGF and OP-1 (50 ng/ml). In the absence of NGF, few neurons (62 ± 25 neurons/well) survived, and this number was not signficantly increased in the presence of OP-1 (93 ± 74 neurons/well). Similar results were obtained with cultures of neurons from 2-day-old pups (data not shown). In addition, OP-1 did not potentiat th effects of submaximal concentrations (0.1–30 ng/ml) of NGF on survival. Thus, the effects of OP-1 are distinct from those of NGF, in that the former does not support neuronal survival.

Comparison of Dendritic Growth in Vitro and in Situ

To assess the potential importance of OP-1 as a regulatory molecule, we compared the amount of dendritic growth that occurs in vitro with that which normally occurs during a comparable period in situ (Table 1). For the former, we used cultures that had been exposed to optimal concentrations of OP-1 and NGF for 2 weeks. For the latter, we used published data (Snider, 1988) describing the dendritic morphology of superior cervical ganglion neurons in 2-week-old rat pups. Cells exposed to OP-1 in vitro were at least as complex as their counterparts in situ. They had about the same number of dendrites as cells in situ, but the length of their dendritic arbor was 38% greater. The increase in the size of the dendritic arbor in vitro seemed to be due primarily to increases in the radial length of dendrites because there was no detectable change in the amount of branching, as assessed by the method of Scholl (1953). Since these experiments were performed in a serum-free medium, it appears that a combination of only two trophic factors is a sufficient stimulus to allow the establishment of a dendritic arbor of approximately normal dimensions.

OP-1 Induces Dendritic Growth in Naive Neurons

Many sympathetic neurons have formed rudimentary dendrites by the time of birth. Therefore, the previous experiments primarily reflect effects of OP-1 on dendritic regeneration. To determine whether OP-1 could also promote de novo formation of these processes, we tested its effects on sympathetic neurons taken from 14.5 day embryos. At this time, there are still many neuroblasts in sympathetic ganglia (Hendry, 1977).

Approximately half of the neurons from control and OP-1-treated cultures synthesized DNA (Table 2). Only a small subpopulation of control neurons formed dendrites; in contrast, 95% of the neurons exposed to OP-1 formed such processes. Moreover, in OP-1-treated cultures, the number of dendrites per neuron was similar in thymidine-labeled and unlabeled cells, suggesting that primitive neurons just exiting the cell cycle respond to OP-1 about as well as more mature postmitotic neurons. OP-1-treated cultures from 14.5 day embryos were examined by immunocytochemistry, and the pattern of staining was essentially identical to that observed in cultures from perinatal animals: the dendrites were selectively labeled with MAbs to MAP2 and nonphosphorylated forms of the M and H neurofilament subunits, while axons reacted with antibodies to tau, synaptophysin, and phosphorylated forms of the M and H neurofilament subunits.

Discussion

Our data indicate that OP-1 induces the formation of dendrites in sympathetic neurons, and that in the presence of an optimal concentration of NGF, the dendritic arbor expands at a rate at least equivalent to that bserved dur-

Table 1. Comparison of the Dendritic Arbors Generated by Sympathetic Neurons in Situ and After a 2 Week Exposure to OP-1

Parameter	In Situ[a]	In Vitro
Number of dendrites/cell	6.9 ± 2.1	7.6 ± 0.4
Total linear length of dendritic arbor (μm)	779 ± 288	1078 ± 77
Maximum extent of arbor (μm)	99 ± 31	171 ± 6
Number of branches crossing 50% circle	8.0 ± 2.8	9.3 ± 0.7
Soma diameter (μm)	29.2 ± 6.0	32.9 ± 0.5

Cultures of sympathetic neurons were immunostained with MAb SMI-32 after a 2 week exposure to OP-1. Data are expressed as the mean ± SEM (n = 40).
[a] In situ data are from Snider (1988). Mean ± SD.

ing the first 2 postnatal weeks in situ. These observations suggest the existence of a novel type of trophic influence upon sympathetic neurons: one that affects cell shape without affecting survival.

Som neural crest derivatives, such as sensory and many parasympathetic neurons, form only axons, whereas others, such as sympathetic neurons, also extend dendrites. The mechanisms that cause sympathetic neurons to diverge in shape have remained obscure. Our data indicate that sympathetic neurons form only axons when they are maintained in the presence of their target-derived survival factor, NGF. Since cells grown under these conditions have previously been shown to have many properties expected of sympathetic neurons, such as appropriate electrical properties, receptors, and neurotransmitter phenotyp (Higgins et al., 1991), it appears that the extension of dendrites is not a constitutive part of the program for sympathetic differentiation. Rather, it seems to be a subroutin whose expression is differentially regulated. In this respect, it is interesting to note that there has been an evolutionary change in the morphology of sympathetic neurons: amphibian sympathetic neurons are typically unipolar while their mammalian counterparts usually have d ndrites (Elfvin, 1983).

Previous studies of sympathetic neurons suggested that the growth requirements of dendrites are more demanding than those of axons, with the former requiring molecules derived from sera, glia, or basement membranes (Tropea et al., 1988; Lein and Higgins, 1989). However, the poorly defined nature of these stimuli made it difficult to distinguish specific effects on dendrites from nonspecific changes in the health of neurons. The current data indicate that exposure to a recombinant growth factor induces dendritic growth, and that this effect is blocked by MAbs that react with OP-1 but not with other closely related BMPs. The effects of OP-1 were observed in cultures grown in the absence of nonneuronal cells, indicating a direct effect upon neurons. Moreover, the structural requirements for inducing dendritic growth are quite stringent since this effect was not observed with 26 other growth factors, including members of the TGFβ family. These observations strongly suggest that specific trophic interactions can regulate the development of neuronal cell shape.

Table 2. OP-1 Induces Dendritic Growth in Sympathetic Neurons from 14.5 Day Embryos

Condition	Nuclear Label	Neurons with Dendrites (%)	Dendrites/ Cell
Control	Yes (45%)	13	0.22 ± 0.09
	No (55%)	7	0.09 ± 0.05
OP-1	Yes (52%)	96	4.62 ± 0.32
	No (48%)	94	4.06 ± 0.32

Sympathetic ganglia from 14.5 day embryos were labeled for 18 hr with [^{3}H]thymidine and then dissociated. After nonneuronal cells had been eliminated, some cultures were treated with OP-1 (100 ng/ml) for 12 days. Cultures were then immunostained with MAb SMI-32 and processed for autoradiography. Data are expressed as mean ± SEM (n ≥ 100).

Most of the sympathetic neurons that were obtained from perinatal pups had established rudimentary dendritic arbors in situ (Voyvodic, 1987). In contrast, ~50% of the neurons taken from 14.5 day embryos were still synthesizing DNA, and the others had just reached the stage where they were beginning to form dendrites in situ (Rubin, 1985). Yet virtually all of the neurons from these two groups formed dendrites in the presence of OP-1. These data suggest a potential morphogenetic role for OP-1 since it not only sustains dendritic regeneration but also promotes de novo synthesis in naive neurons.

The dendrites formed in the presence of OP-1 were appropriate to sympathetic neurons in their shape, length, and number. They also exhibited appropriate functional specializations (Craig and Banker, 1994). Thus, these dendrites accumulate and postranslationally modify selected cytoskeletal and membrane proteins; exclude axonal proteins, transport ribosomes, and other types of RNA (unpublished observations, P. L. and D. H.); and form synaptic contacts of correct polarity. The simplest explanation for these phenomena is that OP-1 induces the execution of a cellular program that controls both quantitative and qualitative aspects of dendritic growth. Consistent with the notion of a program is the recent finding that nodose neurons, which normally do not form dendrites in situ, appropriately segregate cytoskeletal proteins when dendritic growth is induced in vitro (de Koninck et al., 1993). However, if this is the case, it is important to note that this does not appear to be an unrestricted program for dendritic growth, because we never saw sympathetic cells with dendritic arbors that resembled those of pyramidal or Purkinje cells or with well-developed dendritic spines.

NGF regulates dendritic development in sympathetic neurons in situ (Snider, 1988; Ruit t al., 1990). A similar NGF dependence was not d In this study. In fact, in th

presence of optimal concentrations of NGF and OP-1, a dendritic arbor of normal dimensions was formed. Since our experiments were not performed at clonal density, one cannot rule out the involvement of intimate cell–cell interactions, such as those mediated by cell adhesion molecules, in the development of dendrites in vitro. However, since the neurons were grown at low density in the absence of nonneuronal cells, the data suggest that the number of trophic substances that would have to be provided by other cell types, including targets, glia, and preganglionic fibers, is quite limited and that it could potentially consist of only two molecules.

The relevance of this model to the regulation of dendritic growth in situ is still unclear. However, OP-1 mRNA or protein has been detected in most adult rat tissues that receive a sympathetic innervation, with particularly high levels being found in the kidney and in association with basement membranes (Ozkaynak et al., 1992; Wozney, 1993; Vukicevic et al., 1994). OP-1 might therefore function as a target-derived factor. Alternatively, the situation may be more complex, with local sources of dendrite-promoting molecules also being involved. OP-1 and BMP-6 are closely related homologs with greater than 90% amino acid identity in the cysteine portion of the mature protein. Wall et al. (1993) found that BMP-6 is present in high concentration in most parts of the embryonic peripheral nervous system. In addition, Schluesener et al. (1995) have reported that adult rat Schwann cells express BMP-6, and our preliminary data suggest that their embryonic counterparts synthesize OP-1 (P. L. and D. H.). Thus, although the current data are limited, they suggest that sympathetic neurons may be exposed to dendrite-promoting BMPs both during development and in adulthood.

Trophic factors can affect many aspects of the development of sympathetic neurons, including their survival, rates of axonal growth, and neurotransmitter phenotype (Patterson and Nawa, 1993). The actions of OP-1 are distinct from those of NGF (Thoenen and Barde, 1980) and NT3 (Birren et al., 1993) because OP-1 does not support neuronal survival. The effects of OP-1 are also different from those of extracellular matrix molecules such as laminin and collagen IV, which selectively promote axonal elongation (Lein and Higgins, 1989; Lein et al., 1991) and which, in some cases, can also cause the formation of multiple axons. In contrast, OP-1 did not have detectable effects on either the initial extension of axons or the number of axons per cell in long-term culture. The relationship between the regulation of neurotransmitter phenotype and morphological development is less clear. Molecules such as cholinergic differentiation factor/leukemia inhibitory factor and ciliary neurotrophic factor, which induce cholinergic function in sympathetic neurons (Patterson and Landis, 1992), failed to promote dendritic growth, suggesting these developmental processes can be regulated independently. On the other hand, the recent finding that BMP-2 and BMP-6 cause sympathetic neurons to express unique profiles of mRNAs for neuropeptides (Fann and Patterson, 1994) indicates that there could also be some overlap between the regulation of cell shape and neurotransmitter phenotype. In either case, the data are consistent with a model in which multiple trophic interactions govern the expression of the complex assemblage of traits that comprise the sympathetic neuronal phenotype (Patterson and Nawa, 1993). The data also indicate that members of the BMP/OP family can exert profound and unique effects on the development of autonomic neurons. Such actions are consistent with their well-established role as differentiation factors in other systems, especially mesenchymal tissues (Wozney, 1993).

Experimental Procedures

Materials

Mature human recombinant OP-1 was isolated from medium conditioned by transfected Chinese hamster ovary cells using S-Sepharose and phenyl-Sepharose chromatography followed by reverse phase high performance liquid chromatography (Sampath et al., 1992). The purity was >98%, as assessed by SDS–polyacrylamide gel electrophoresis. Other growth factors were obtained from commercial sources: GIBCO/BRL (interleukin-1β, -3, -4, -6, and -7; epidermal growth factor, basic fibroblast growth factor, granulocyte/macrophage colony-stimulating factor, leukemia inhibitory factor, TGFα, and rat Γ interferon); PeproTech, Inc. (brain-derived neurotrophic factor, NT3, NT4, ciliary neurotrophic factor, and glial-derived neurotrophic factor); R&D Systems (TGFβ1, TGFβ2, TGFβ3, and vascular endothelial growth factor); Collaborative Biomedical Products (hepatocyte growth factor, platelet-derived growth factor, and TGFβ); Boehringer Mannheim (interleukin-2); and Promega (interleukin-8). MAbs (1B12 and 12G3; Creative Biomolecules, Inc.) that react with OP-1, but not with BMP-2 or BMP-4, were affinity purified with Protein A agarose (Vukicevic et al., 1994).

Tissue Culture

Suspensions of neurons dissociated from the superior cervical ganglia of Holtzman (Harlan Sprague–Dawley) rat fetuses (20–21 days) or rat pups (1–3 days postnatal) were prepared according to the method of Higgins et al. (1991). Equivalent results were obtained with pre- and postnatal animals. Neurons were plated at low density (~10 cells/mm²) onto poly-D-lysine-coated (100 μg/ml) coverslips and maintained in a serum-free medium (Higgins et al., 1991) containing β-NGF (100 ng/ml) and three other proteins: bovine serum albumin (500 μg/ml), bovine insulin (10 μg/ml), and human transferrin (10 μg/ml). Equivalent results were obtained with NGF purified from mouse salivary glands (gift from E. M. Johnson) and recombinant human NGF (PeproTech, Inc.). Cytosine-β-D-arabinofuranoside (1 μM) was added to the medium of all cultures for 48 hr beginning on the second day; this exposure was usually sufficient to render them virtually free of nonneuronal cells for 30 days.

To label sympathetic neuroblasts, ganglia from 14.5 day rat embryos were grown in explant culture for 18 hr in the presence of [methyl-³H]thymidine (0.3 μCi/ml; ICN Biomedicals, Inc.). Subsequently, the ganglia were exposed to trypsin, and the dissociated cells were plated on laminin-coated (10 μg/ml) coverslips (Higgins et al., 1991). Because NT3 (50 ng/ml) enhances the survival of immature sympathetic neurons (Birren et al., 1993), it was added to the NGF-containing medium during the period of explant culture and during the first 4 days of dissociated cell culture.

Morphological Analyses

Cellular morphology was routinely assessed by the intracellular injection of fluorescent dyes, Lucifer yellow (4%) or 5(6) carboxyfluorescein (5%). Only neurons whose cell bodies were at least 150 μm from other neuronal somata were injected because density-dependent changes in morphology occur when sympathetic neurons are separated by lesser distances (Bruckenstein and Higgins, 1988).

Cultures were immunostained with antibodies previously shown to react selectively with either axons or dendrites (Sternberger and Ster-

nberger, 1983; Wiedenmann and Franke, 1985; Binder et al., 1986; Lein and Higgins, 1989; Cameron et al., 1991). MAbs to MAP2 (AP13 and AP14; gift of L. I. Binder), to nonphosphorylated forms of the M and H neurofilament subunits (SMI-32; Sternberger–Meyer Immunocytochemicals), and to the transferrin receptor (MRC OX-26; Serotech) were used as dendritic markers. Axonal probes included MAbs to synaptophysin (SY-38; Boehringer Mannheim), tau (Tau1; L. I. Binder), and phosphorylated forms of the H (NE14; Boehringer Mannheim) and the M and H (SMI-31; Sternberger–Meyer Immunocytochemicals) neurofilament subunits. All antigens were localized by indirect immunofluorescence using previously described procedures (Lein and Higgins, 1989). Image 1 Software (Universal Imaging) was used to quantitate dendritic growth in immunostained cultures. Maximum dendritic extent was determined by measuring the radius of a circle encompassing the entire arbor. The number of branches crossing a circle of half that diameter was used as an index of branching (Scholl, 1953). Cultures were prepared for electron microscopy according to published procedures (Tropea et al., 1988). Data in the text are presented as mean ± SEM.

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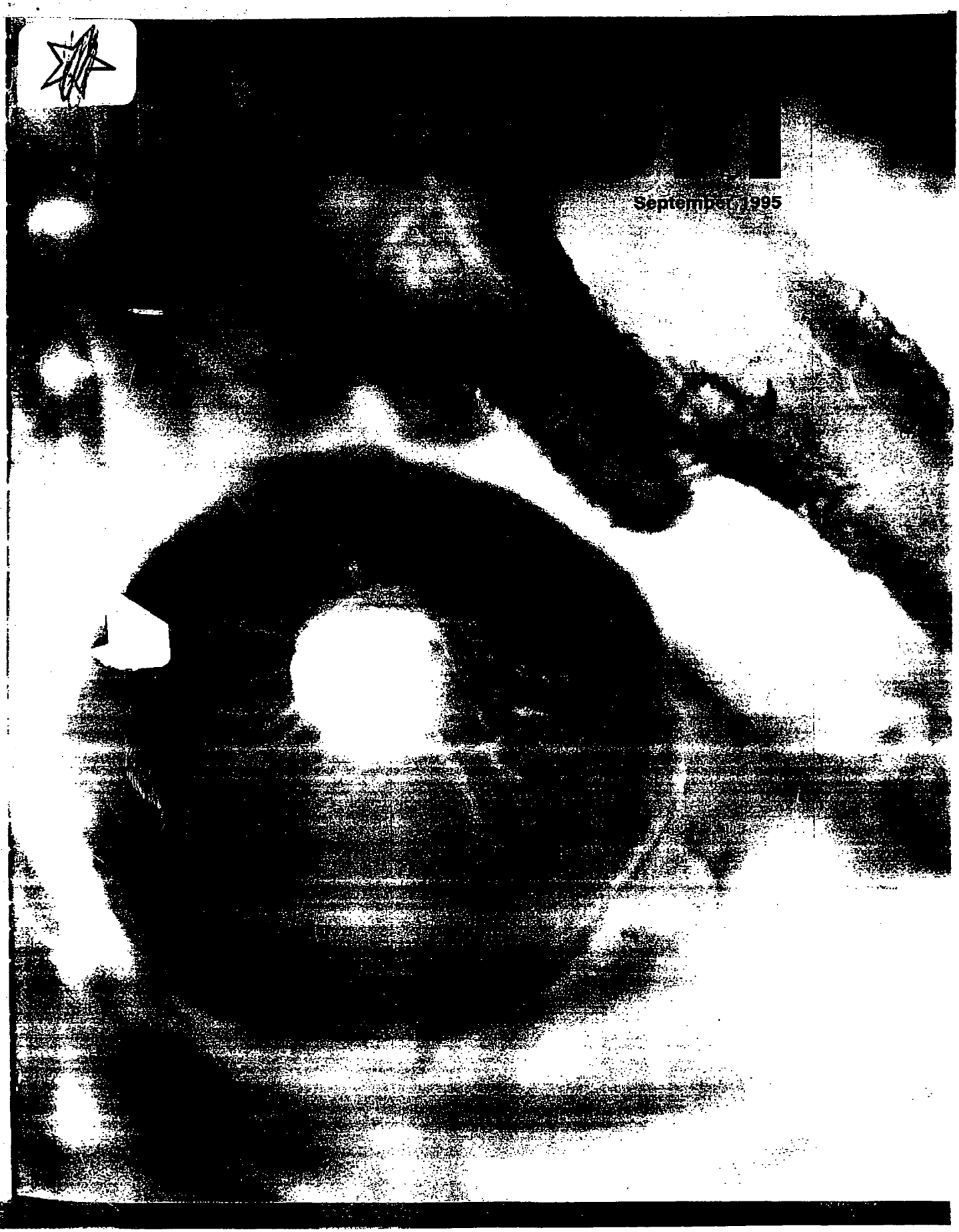

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Effects of Osteogenic Protein-1 (OP-1) Treatment on Fetal Spinal Cord Transplants to the Anterior Chamber of the Eye

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Spinal cord injury represents a serious medical problem, and leads to chronic conditions that cannot be reversed at present. It has been suggested that trophic factor treatment may reduce the extent of damage and restore damaged neurons following the injury. We have tested the effects of osteogenic protein-1 (OP-1, also known as BMP-7), a member of the transforming growth factor-β superfamily of growth factors, on developing spinal cord motor neurons in an intraocular transplantation model. Embryonic day 13 or 18 spinal cord tissue was dissected, incubated with OP-1 or vehicle, and injected into the anterior chamber of the eye of adult rats. Injections of additional doses of OP-1 were performed weekly, and the overall growth of the grafted tissue was assessed noninvasively. Four to 6 weeks postgrafting, animals were sacrificed and the tissue was processed for immunohistochemistry using antibodies directed against choline acetyltransferase, neurofilament, and the dendritic marker MAP-II. We found that OP-1 treatment stimulated overall growth of spinal cord tissue when dissected from embryonic day 18, but not from embryonic day 13. OP-1 treatment increased cell size and extent of cholinergic markers in motor neurons from both embryonic stages. The neurons also appeared to have a more extensive dendritic network in OP-1-treated grafts compared to controls. These findings indicate that OP-1 treatment may reduce the extent of axotomy-induced cell death of motor neurons, at least in the developing spinal cord.

Key words: Spinal cord; Development; Transplantation; Trophic factors; Bone morphogenic factors; Acetylcholine; Motor neurons; Transforming growth factor-β

INTRODUCTION

Members of the transforming growth factor-β (TGF-β) superfamily of proteins, including activins and bone morphogenetic proteins (BMPs), comprise an evolutionarily well-conserved group of proteins that controls differentiation, cell growth, and morphogenetic processes in a number of different systems during development (30,36). The BMPs, which comprise a subgroup of the TGF-βs, have been shown to affect embryonic skeletal development (9,37), musculoskeletal neoplasms (41), tooth formation, in particular dentinogenesis (23), as well as renal branching morphogenesis (8,34). Osteogenic protein-1 (OP-1, also known as BMP-7) was first isolated from extracellular bone matrix, based on its ability to induce new bone formation in vivo (36). Members of the TGF-β superfamily encode secreted proteins that share common structural features. They are proteolytically processed from the precursors to yield a carboxy-terminal mature protein varying from 110 to 150 amino acids. All members share a conserved pattern of cysteines in this domain. The active ligand form is a disulfide-bonded homodimer of a single family member or a heterodimer of two different members [see (16,30, 31)]. In comparison to other TGF-β superfamily members, OP-1 was shown to differentially regulate progression of the osteogenic lineage (9). A number of studies have shown that OP-1 can induce the formation of new bone when placed in a bony environment (9,12, 36,37).

The TGF-βs and BMPs selectively signal to the responding cells through oligomeric complexes of type I and type II serine/threonine kinases (27,32,39). It was recently demonstrated that cells in the central nervous system (CNS) express high levels of BMP receptor type II, suggesting that BMPs may serve functions in the mature CNS. During early development of the neural systems, BMPs have been shown to play a role in neurulation and dorsoventral patterning of the neural tube as well as promoting the generation of astrocytes and radial glia [(6,17,18); see also (32)]. During later stages of

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neural differentiation, BMPs potentiate the survival of regional neuronal phenotypes and enhance the outgrowth of dendritic processes (24,25,32,35,42). The specific role of OP-1 versus other BMPs in these processes has not yet been elucidated. OP-1 has, for example, been shown to induce dendritic growth in cultured sympathetic neurons (24). These authors reported that OP-1 treatment of these cultures elicited an extensive dendritic response that correctly segregated and formed synaptic contacts of appropriate polarity (24). With the addition of nerve growth factor (NGF) to the culture, OP-1 induced dendritic growth on perinatal sympathetic neurons comparable to that observed in situ. These findings suggested that OP-1 might also be used as a dendritic growth promoter for other neuronal populations, such as the dorsal root ganglion or spinal cord motor neurons. Indirect evidence for OP-1 effects on spinal cord development was provided by Söderström et al. (39), who demonstrated that mRNA for the OP-1 receptor BMPR-II was present in spinal cord during embryonic development. These authors demonstrated strong BMPR-II mRNA expression in spinal cord, dorsal root ganglia, and spinal nerves at embryonic day 16, and with marked labeling maintained in the spinal cord and dorsal root ganglia at embryonic day 21 in the rat (39). These findings suggest that spinal cord neurons would be responsive to treatment with OP-1, at least during embryonic development.

Classic work using animal models and tissue culture has identified many different trophic molecules that have effects on spinal cord motor neuron survival and/or differentiation. For example, several members of the neurotrophin family of trophic factors have been found to stimulate spinal cord neuronal survival and differentiation, including brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) [see (7,10,11,19)]. Ciliary neurotrophic factor (CNTF) has also been found to prevent degeneration of adult motor neurons after axotomy (5,38). The members of the neurotrophin family have so far been the best candidates for treatment of spinal cord injury in the clinic, but, surprisingly, inactivation of their receptor genes leads to only partial loss of motorneurons, suggesting that other factors may be involved in motor neuron survival during development and repair [see, e.g., (5)]. We and others have previously shown that TGF-β superfamily members can promote survival and differentiation of spinal cord motor neurons, both in culture and in vivo. Glial cell line-derived neurotrophic factor (GDNF), another member of the TGF-β superfamily of growth factors (28), was found to support the survival of purified rat motor neurons in culture (20,43) as well as to prevent axotomy-induced cell death in adult spinal cord and brain stem motor neurons (20,26). In addition, we have previously shown that GDNF, when injected directly into the intraocular fluid (40) or administered using a novel carrier-mediated transport with transferrin receptor conjugates (1), can enhance survival of spinal cord tissue transplanted to the anterior chamber of the eye. The intraocular transplantation technique has been used extensively to evaluate trophic factor efficacy, because tissue can be observed noninvasively and longitudinally to assess growth and vascularization [see (2,14,40)]. We have demonstrated that CNS tissue will survive transplantation and develop normal vascularization with a functional blood–brain barrier (15) and that spinal cord transplants develop many physiological and morphological characteristics of the mature spinal cord neurons in situ (21,22).

The studies of GDNF effects on spinal cord transplants and the temporo-spatial distribution of OP-1 and its receptors (39) prompted us to investigate the effects of OP-1 on spinal cord transplants. The specific aim of the present study was to determine if OP-1 could enhance survival of fetal spinal cord tissue in the anterior chamber of the eye. We utilized tissue from two different fetal stages, embryonic day 13 and 18, to determine whether we could expand the developmental window for transplantation of spinal cord tissue. Fetal spinal cord tissue is known to survive transplantation only when obtained from immature developmental stages, earlier than embryonic day 15 in the rat (22).

MATERIALS AND METHODS

Transplantation and Intraocular Injections

Spinal cord tissue from two different embryonic stages (embryonic day = E13 and E18), determined by the crown–rump length of the fetus, was dissected from the cervical portion of the spinal cord of fetuses from timed pregnant Fischer 344 rats, as previously described (21). Ten animals with bilateral transplants were used for the E18 donor stage (10 transplants in each group), and 20 animals, performed at two different occasions (10 each time), were used for the E13 donor stage (a total of 20 transplants per treatment group). Prior to intraocular transplantation, the tissue pieces were incubated for 20 min at room temperature with 100 μg/ml of OP-1 (obtained from Creative Biomolecules, Hopkinton, MA). Control tissue pieces were preincubated for the same time period with vehicle solution. Young adult Fisher 344 female rats (150 g, Harlan) were anesthetized with xylazine (Rompun, 12 mg/kg, IP) and ketamine (Ketaset, 80 mg/kg, IP), and their pupils were dilated with one drop of a 1% atropine solution. A small incision was made in the cornea with a razor blade tip and one tissue piece was injected into each eye with a modified syringe (13). Transplants were allowed to mature for 4–6 weeks postgrafting, during which time weekly measurements were made through the translucent cornea

with a stereoscopic microscope to establish growth curves. The measurements represented the area of each transplant, as previously described [see, e.g., (2,13,14)]. Because individual transplant size may vary (tissue pieces of fetal tissue at an exactly similar size are difficult to obtain, especially when using whole slices of spinal cord from two different donor stages), we normalized the transplant growth, so that each value shown in Figure 1 represents the growth of that particular transplant, expressed as increase over initial size (= 1.0). This way, it is possible to evaluate differences between growth of spinal cord from different donor stages, which have different initial size. Beginning 1 week postgrafting, weekly intraocular injections of the same compounds were performed (1 μg of OP-1 per injection in 5 μl vehicle). The dosage chosen for OP-1 preincubation and intraocular injections was based upon previous work using GDNF and intraocular spinal cord grafts (40), as well as previous data on OP-1 treatment of neurons in tissue culture (24,25,35,42). Statistical analysis of the transplant size was performed using weeks 0–4 as repeated measures (ANOVA) to determine the growth rate over time. The curves were fit by polynominal regression and the Scheffe test was employed to account for the multiple comparisons made at each of the weeks.

Immunohistochemistry

Transplants were dissected from the host iris and processed for immunohistochemistry to determine the appearance of motor neurons in the spinal cord tissue. The transplants were immersion fixed overnight in paraformaldehyde (4%). They were transferred to 30% sucrose in phosphate buffer (PB) for at least 16 h, after which they were sectioned at 8–12 μm on a cryostat (Zeiss/Microm, Carl Zeiss Inc., Tempe, AZ). Every 10th section was collected for routine histology, using cresyl violet staining on glass slides. The sections used for immunohistochemistry were mounted on slides and rinsed in phosphate-buffered saline (PBS) containing 0.05% Triton X-100. Sections were then incubated 48 h with antibodies directed against choline acetyltransferase (ChAT, Boehringer-Mannheim, dilution 1:20), the dendritic marker MAP-II (Sigma, 1:100), and neurofilament (NF, 1:100, Dakopatts). The antibodies were diluted in PBS with 1% normal goat serum and 1% serum albumin to reduce background staining. The sections were rinsed in PBS and incubated with anti-rabbit (ChAT and MAP-II) or antimouse (NF) IgG conjugated to fluorescein isothiocyanate (FITC, 1:100; Vector Laboratories Inc., Burlingame, CA). They were studied in a Nikon Optiphot microscope (Nikon, Tokyo, Japan). Specificity controls included sections where the first or second antibody was mitted. When the terms "-immunoreactive" or "-positive" are used in the text, this always means "like immunoreactivity." because the indirect immunohistochemistry techniques used in the study cannot directly verify the identity of tissue antigens.

Image Analysis

The sections labeled with ChAT, NF, and MAP-II antibodies were studied in terms of overall staining intensity and cell size, using an image analysis system distributed by the National Institutes of Mental Health (NIH Image). The Nikon Optiphot microscope was connected to a Macintosh Quadra computer by means of a Cohu analog video camera (model 4990) and an analog/digital card. Images were collected with the video camera using the 10× objective and measurements of overall staining intensity and the average size of immunoreactive neurons were obtained from every fifth section for each antibody throughout each transplant. The staining intensity measurements were analyzed after background fluorescence had been subtracted from each section, to control for differences that might occur due to different thickness of individual sections, etc. The mean size and staining intensity was determined for each animal and the mean of means used as a group value for the two different groups examined. Statistical analysis of measurements was performed using two-way Student's *t*-tests. For further details on this image analysis system see Bäckman et al. (3) and Bowenkamp et al. (4).

RESULTS

Overall Growth of Spinal Cord Transplants

When tissue from E18 was used, there was a significant difference in the growth of spinal cord tissue in response to OP-1 (Fig. 1a). Very few control grafts contained surviving tissue at all after 2 weeks in oculo, compared to the OP-1-treated group where a more robust survival was seen (an anterior eye chamber with only remnants of a control graft is shown in Fig. 2b, versus a healthy and fairly large transplant, treated with OP-1, in Fig. 2a). As can be seen in Figure 1, the OP-1-treated grafts grew significantly larger than the controls throughout the experiment. Only 50% of the transplants (5 of 10 total transplants) survived at all in the control group from donor age E18, whereas 90% (9 of 10 transplants) survived in the OP-1-treated group. Statistical analysis with multiple comparisons (ANOVA with Scheffe test) revealed significance between the two groups in terms of overall tissue growth at 2, 3, and 4 weeks postgrafting (see Fig. 1a). In additional experiments, we transplanted spinal cord tissue from E13 and treated the tissue with either OP-1 or vehicle. As can be seen in Figure 1b, there was no significant difference in terms of overall tissue growth between the groups in transplants from donor stage E13. From this donor stage, 80% of the transplants in the control group survived (16

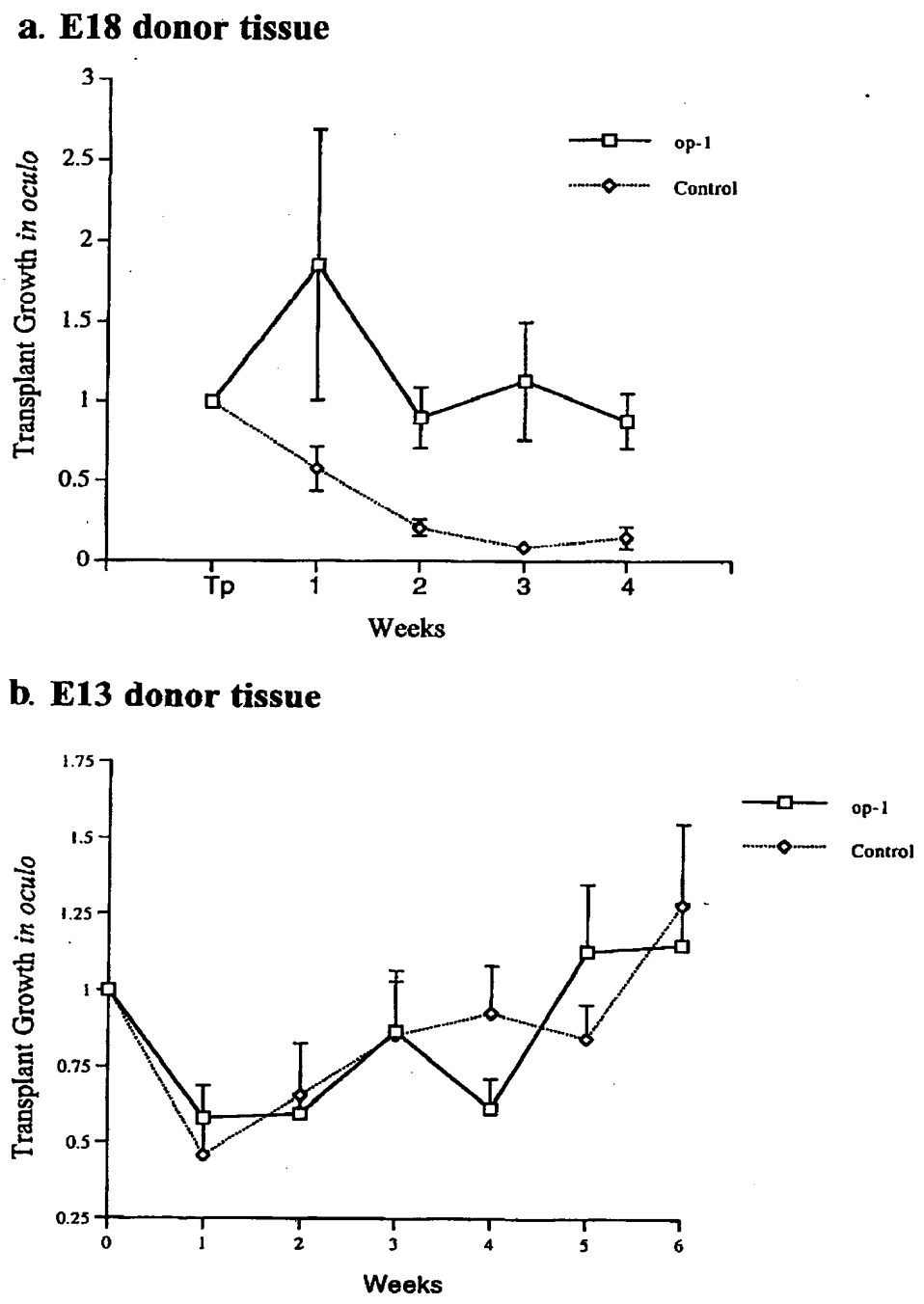

Figure 1. Growth curves for intraocular spinal cord transplants. (a) Embryonic day 18 donor stage; (b) embryonic day 13 donor stage (E18 = embryonic day 18; E13 = embryonic day 13). The *x*-axis represents time in weeks from grafting (= 0, Tp), and *y*-axis represents transplantation growth from input size (= 1). Repeated measures ANOVA revealed a difference between the groups in (a), but not in (b) ($p < 0.05$).

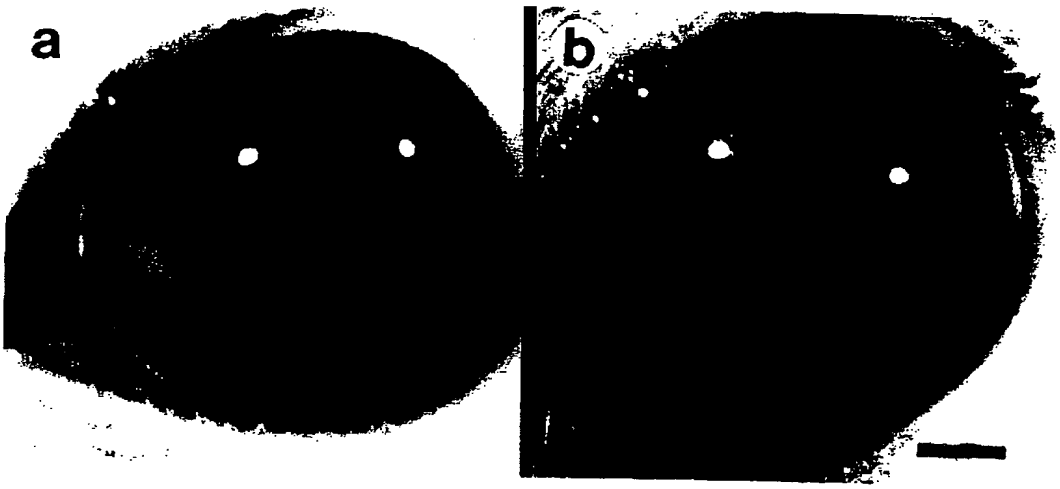

Figure 2. Intraocular spinal cord grafts from embryonic day 18. (a) OP-1-treated graft; (b) control graft. These two grafts are representative for the two groups, because only 50% of the grafts in the control group survived. Scale bar represents 1 mm.

of 20 transplants totally, see Fig. 1b), and 100% of the E13 transplants survived with OP-1 treatment (20 of 20 transplants).

General Histology

Transplants from both groups were sectioned and prepared for routine histology using cresyl violet staining. The OP-1-treated grafts appeared to contain a greater density of large neuronal cell bodies than control grafts (Fig. 3). Quantitative image analysis of cell body size revealed a 45% increase in the mean diameter of neuronal cell bodies in the OP-1-treated groups compared to controls (mean ± SEM values for overall cell size were 355 ± 26 μm^2 for the OP-1 group; $n = 9$, and 245 ± 45 μm^2 for the control group; $n = 5$). Statistical analysis between the two different groups (two-way Student's *t*-test) revealed a significant difference at the level of $p < 0.05$. Other components of the transplants appeared normal and similar between the two groups, such as vascularization.

Immunohistochemistry

Image analysis of sections stained with immunohistochemistry did not reveal any differences in terms of overall staining densities between the OP-1 groups from E13 or E18 donor stages, or between controls grafts from these two different stages. Due to the poor survival rate of control transplants, the different image analysis mean values were therefore pooled between the two control groups, from E13 and E18, as well as the two OP-1 groups, from E13 and E18. However, incubation of the transplanted tissue with antibodies directed against ChAT revealed a significant difference in both appearance and density of staining between groups of animals treated with OP-1 versus control solution, regardless of donor stage (Fig. 4a–d). The same magnitude of difference between the OP-1-treated group and the control group was seen at both the E13 and the E18 donor stages. Moreover, individual neurons were larger and exhibited significantly more extensive neurite branching. This was confirmed by quantitative image analysis of the overall ChAT staining density (Fig. 5a). The mean ± SEM staining density was 1.45 ± 0.04 for the OP-1-treated group ($n = 7$; staining/background ratio; $p < 0.01$) and 1.21 ± 0.07 for the control group ($n = 6$). In contrast to the enhanced cholinergic markers, there was no statistical difference between the two groups, with respect to staining density for neurofilament, even though there was a trend towards an increase in the OP-1 group (Figs. 5b and 6a, b). The mean ± SEM staining/background ratio for neurofilament immunohistochemistry was 1.20 ± 0.05 for the OP-1 group ($n = 7$) and 1.04 ± 0.01 for the control group ($n = 6$). Sections were also stained for the dendritic marker MAP-II (Figs. 5c and 6c, d). There was a significant difference in the appearance, but not in staining density, of MAP-II-immunoreactive profiles in the two groups (see Fig. 6c, d). When overall staining density measurements were performed over the whole transplant area, there was no difference in the staining/background ratio of MAP-II staining (Fig. 5c). The mean ± SEM value for MAP-II staining was 1.19 ± 0.01 ($n = 7$) in the OP-1 group, and

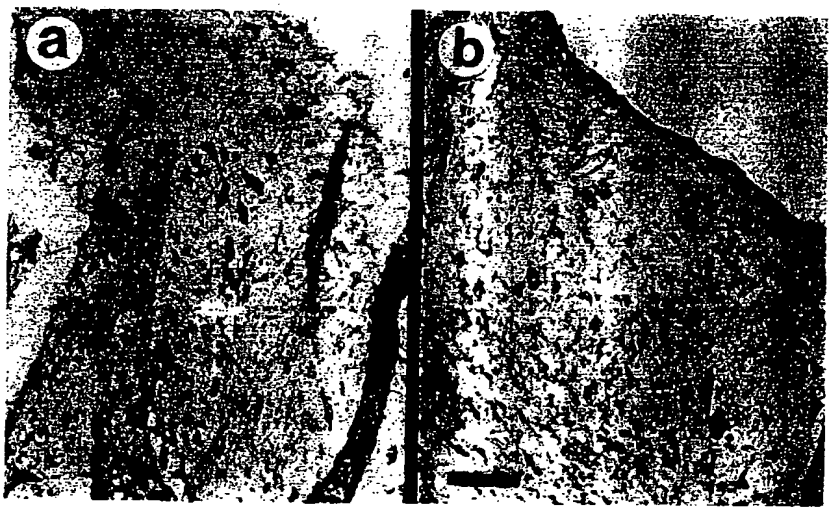

Figure 3. Cresyl violet-stained sections of spinal cord grafts. (a) OP-1-treated graft, (b) control graft. Note the difference in number and size of large motor neurons between the two groups. Scale bar represents 75 μm.

1.17 ± 0.01 in the control group ($n = 6$). Because the dendritic branches are only present in smaller portions of the spinal cord grafts (the gray matter of the ventral horn), it is not surprising that unbiased measurement of staining density resulted in similar mean values for the two groups. However, as can be seen in Figure 6, there was a qualitative difference in the appearance of MAP-II staining between OP-1-treated grafts and controls. The areas with significant dendritic branching in OP-1-treated grafts (Fig. 6c) contained a higher density of MAP-II-immunoreactive profiles, which also appeared to be more developed in terms of thickness and branching, than similar areas in the vehicle-treated grafts (Fig. 6d).

DISCUSSION

We have demonstrated that OP-1 stimulates cholinergic and dendritic markers in embryonic spinal cord tissue grafted into the anterior chamber of the eye of the adult rat. There was an increase in the overall graft survival of spinal cord transplants with OP-1 when donor tissue was obtained from E18, but not when transplants were obtained from E13. The number of surviving transplants in the OP-1-treated group was significantly greater than in the control group when E18 tissue was used. These findings are in agreement with earlier studies, which have reported enhancement of dendritic processes in sympathetic neurons in culture with OP-1 (25) and also a dendritic growth enhancement in hippocampal cultures (42). We only saw MAP-II staining in portions of the grafts where neuronal cell bodies were located. This is not surprising, because motor neurons appear only in the gray matter of the spinal cord, and the grafted tissue consisted of a complete section of fetal spinal cord tissue. It is difficult to perform an unbiased measurement of dendritic branches in the two groups, and no overall differences could be detected using image analysis of staining densities of MAP-II in whole sections of transplants. However, as demonstrated in Figure 6, we found a qualitative difference in MAP-II staining in areas of neuronal cell bodies in the group treated with OP-1 compared to controls.

It was interesting to note that an OP-1 enhancement of overall tissue growth could be seen in transplants from donor stage 18, and not from E13 transplants. There are several possible explanations for this phenomenon. First, we have previously shown that E18 spinal cord transplants survive poorly when transplanted to the anterior eye chamber (22). In the present study, only 50% of the control grafts survived when donor tissue from E18 was used, compared to 80% survival in the E13 control group. Embryonic day 13 spinal cord tissue has a much greater survival capacity and develops into larger transplants expressing organotypic morphological and physiological features of spinal cord neurons in situ (21,22). Thus, it is likely that the differences in OP-1 response seen between the two different donor stages may be due to the fact that the control grafts in the E13

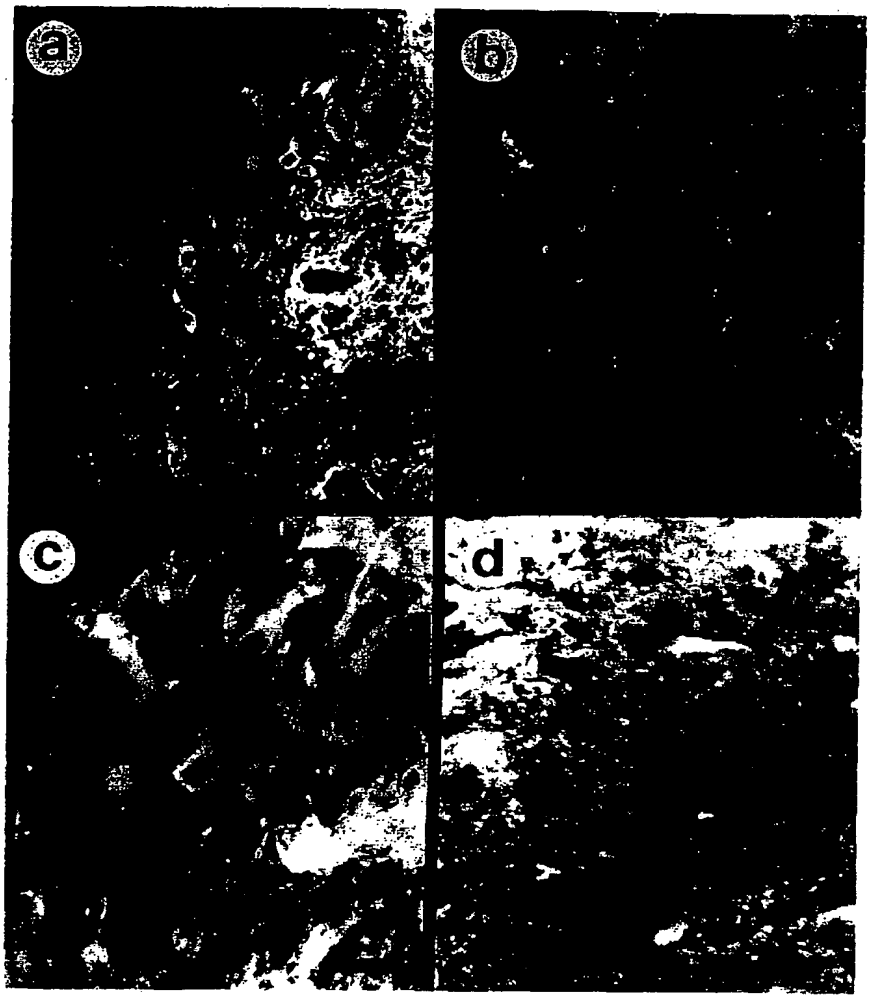

Figure 4. Photomicrographs from sections of spinal cord tissue, incubated with ChAT antibodies. (a, c) OP-1-treated graft; (b, d) control graft. Note that there are very few cholinergic cell bodies present in the control graft, compared to the dense group of motorneurons in the OP-1-treated group. The individual neurons were also larger in the OP-1 than in the control graft. Scale bar represents 30 μm.

group survived better than the controls from E18. Another explanation may relate to the expression of OP-1 receptors during embryonic development. Söderström and collaborators have recently shown that BMPR-II receptor expression increases gradually in the spinal cord during early development and reaches its peak somewhere between E16 and E21 in the rat (39). The intensity of BMPR-II labeling was found to be lower at E11 and E15 than at E16. It is possible that spinal cord transplants contain more receptors at E18 than at E13, and are thus more responsive to OP-1. This hypothesis can be tested in future experiments, with mRNA measurements of the OP-1 type I and II receptors in spinal cord transplants at different donor stages.

The specific signaling mechanisms for OP-1, in terms of dendritic growth and neuronal differentiation, have not yet been elucidated. It has been suggested from tissue culture experiments (33) that OP-1 may act by a transcriptional mechanism of gene regulation that is independent of cell differentiation. Other investigators have shown that NGF and OP-1 have synergistic effects during the development and differentiation of sympathetic neurons in tissue culture (25), suggesting that OP-1 may indeed work in conjunction with other differentia-

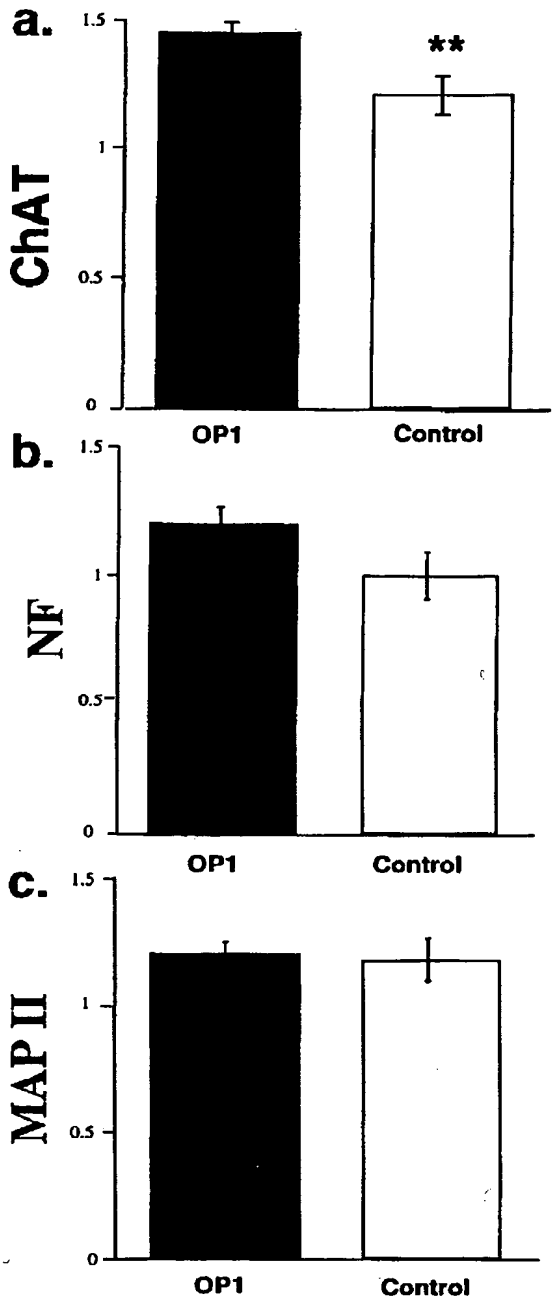

Figure 5. Image analysis results from overall staining densities of ChAT (a), neurofilament (NF; b), and MAP-II (c) immunohistochemistry in entire sections of spinal cord grafts. There was a significant difference between OP-1 and control groups in terms of ChAT immunostaining (a; $p < 0.01$), but there was no significant difference between the two groups in terms of neurofilament staining (b). The overall MAP-II staining density was similar between the two groups (c).

tion factors. Perides and his collaborators (33) found that OP-1 administration to neuroblastoma and glioma hybrid cells gave rise to a significant and long-lasting upregulation of all three maj r isoforms of neural cell adhesion molecules (N-CAM). Because this cell adhesion molecule has been shown to be involved in neurite outgrowth and synaptogenesis [see (29)], it is possible that the dendritic enhancement seen in our study and others may be mediated by the effect on cell adhesion molecules such as N-CAM. It would be interesting to examine the signaling cascades of BMPs and how they interact with the signaling cascades of the adhesion molecules. BMP ligands exhibit cooperative binding to distinct classes of type-I (BMPR-I) and type-II (BMPR-II) receptor subunits with transphosphorylation of the type I subunit by the type II receptor, and subsequent intracellular signaling (32). Different BMPs exhibit distinct profiles of receptor subunit binding; different cell types can display alternative patterns of receptor binding, and thus display a differential response pattern to the different BMPs (32). One current hypothesis for the BMP signaling cascade holds that the activation of the BMPR-I subunit results in recruitment and phosphorylation of SMAD1 (a member of the MAP kinase family), which acts as a latent transcription factor. Activated SMAD1 subsequently associates with SMAD4 and the complex translocates to the nucleus where it associates with gene products to activate developmental stage-specific genes (32). It is not known which developmental genes activate dendritic outgrowth, or if these gene products are closely associated with, for example, adhesion molecule synthesis and/or activation. Nevertheless, further studies are needed to determine if there is a specific BMP signaling cascade in the neuron, separate from those described for traditional growth factors. Because most TGF-βs utilize serine/threonine kinase receptors (27,32,39) as opposed to the tyrosine kinase receptors previously described for the neurotrophins (3,5,7), it is not likely that identical intracellular second messenger cascades are involved in these two different superfamilies of trophic factors, but it is interesting that they may act simultaneously on selective phenotypes of developing neurons.

We have previously found that GDNF stimulates survival of motor neurons in spinal cord transplants (1,40). Because this TGF-β superfamily member appears to exhibit different signaling cascades and different receptor profiles than the BMPs, it would be interesting to perform a combined treatment of spinal cord transplants with both OP-1 and GDNF. This combination may enhance differentiation of the motor neurons (GDNF) as well as dendritic outgrowth and cholinergic markers (OP-1). Such combination treatments are predicted to have more robust effects for clinical purposes than any

Figure 6. Immunohistochemistry for neurofilament (a, b) and MAP-II (c, d). There was a tendency towards an overall increase in neurofilament staining between OP-1 (a) and control grafts (b) as is illustrated in this microphotograph. Overall neurofilament immunoreactivity appeared denser in the OP-1 grafts than in controls. The same pattern was seen with the dendritic marker MAP-II (c, d). Much denser staining for MAP-II was seen in areas of OP-1-treated grafts that contained cell bodies (see left side of c), whereas there was a more sparse staining in controls (d). Scale bar represents 20 μm.

trophic factor alone, because trophic factors exhibit specific and differential effects on separate components of neural development and plasticity. The two factors may act on the same cells or could act on different cells in the same transplant. It would also be interesting to determine if OP-1 is effective in treatment of the adult, injured spinal cord, even though expression of its receptors is known to decrease in late embryonic development (39). Importantly, it is not yet known if OP-1 or its receptors are upregulated in response to injury in the adult spinal cord. These complexities notwithstanding, our data do document a significant positive effect of OP-1 on spinal cord tissues, both in terms of survival and neurite development.

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Leukemia Inhibitory Factor and Ciliary Neurotrophic Factor Cause Dendritic Retraction in Cultured Rat Sympathetic Neurons

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Dendritic retraction occurs in many regions of the developing brain and also after neural injury. However, the molecules that regulate this important regressive process remain largely unknown. Our data indicate that leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF) cause sympathetic neurons to retract their dendrites *in vitro*, ultimately leading to an ~80% reduction in the size of the arbor. The dendritic retraction induced by LIF exhibited substantial specificity because it was not accompanied by changes in cell number, in the rate of axonal growth, or in the expression of axonal cytoskeletal elements. An antibody to gp130 blocked the effects of LIF and CNTF, and both cytokines induced phosphorylation and nuclear translocation of stat3. Moreover, addition of soluble interleukin-6 (IL-6) receptor to the medium endowed IL-6 with the ability to cause dendritic regression. These data indicate that ligands activating the gp130 pathway have the ability to profoundly alter neuronal cell shape and polarity by selectively causing the retraction of dendrites.

Key words: leukemia inhibitory factor; ciliary neurotrophic factor; osteogenic protein-1; bone morphogenetic protein; dendritic retraction; dendrites; sympathetic neurons; stat3; gp130

Dendritic retraction takes many forms in the developing nervous system. In the magnocellular nucleus of chick embryos, neurons initially extend several long, branched dendrites. They then retract all of these processes as they become unipolar (Jhaveri and Morest, 1982). Purkinje cells also extend multiple primary dendrites; however, only the apical process survives, allowing these cells to assume their characteristic shape (Armengol and Sotelo, 1991). In other cases, dendritic regression is less dramatic, but the consequences are still important. For example, all the pyramidal cells in layer 5 of the visual cortex initially form apical dendrites that extend to layer 1 (Koester and O'Leary, 1992). However, as they mature, neurons whose axons have entered the corpus callosum retract the segments of their dendrite that contact the three most superficial cortical layers, thereby becoming short pyramidal cells. In contrast, neurons that send their axons to the tectum do not experience regression of the apical process and so become tall cells. Because different afferent fibers course through the various cortical layers, it is thought that this differential retraction leads to alterations in synaptic function. Dendritic retraction also occurs in many other parts of the developing brain, including the hippocampus (Rihn and Claiborne, 1990), lateral geniculate nucleus (Leuba and Garey, 1984), and autonomic ganglia (Landmesser and Pilar, 1974). In addition, dendritic atrophy has been observed during normal aging (Flood, 1993), in degenerative conditions, including Alzheimer's (Flood and Coleman, 1990) and Parkinson's diseases (Patt et al., 1991), and after acute neural injury (Sumner and Watson, 1971; Yawo, 1987). Thus, regressive events within dendritic processes are widespread, and they are important determinants of neuronal cell shape and synaptic connectivity.

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Several studies suggest that deprivation of target-derived growth factors contributes to the dendritic retraction that is induced by axotomy (Yawo, 1987; Purves et al., 1988; Snider, 1988). In addition, excitatory amino acids such as glutamate have been found to cause dendritic retraction in some types of cultured neurons (Metzger et al., 1998), and there is evidence that these neurotransmitters are involved in certain forms of injury and stress-induced dendritic regression *in vivo* (McEwen and Magarinos, 1997). These observations suggest that there may be multiple pathways that produce dendritic atrophy. However, mediators f dendritic retraction have been identified in only a limited number of systems, and so in most instances we do not know which molecules regulate this important regressive process.

Leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF) belong to the neuropoietic family of cytokines; other family members include interleukin-6 (IL-6), IL-11, oncostatin-M (OSM), and cardiotrophin-1 (Patterson, 1994). Neuropoietic cytokines are expressed in many regions of the nervous system, as are their receptors. Sympathetic neurons are one of the classes of neurons that express receptors for these cytokines, and it is known that they are exposed to several family members, both during their development and after neural injury. This has led to the use of these neurons as a model system for analyzing the effects of neuropoietic cytokines (Patterson, 1994; Landis, 1996; Zigmond et al., 1996; Mehler and Kessler, 1997). The response of sympathetic neurons to these agents is complex and includes changes in the neurotransmitter phenotype as well as in cell survival and the expression of transmitter receptors. In addition, it has previously been observed (Guo et al., 1997) that neuropoietic cytokines inhibit the initial extension of dendritic processes in cultures of sympathetic neurons that have been treated with osteogenic protein-1 [(OP-1) also known as bone morphogenetic protein-7]. In this study, we extend these observations by examining the effects of LIF and related cytokines on existing dendrites. Our data indicate that LIF and CNTF have the ability to

selectively modify the shape of sympathetic neurons by inducing the retraction of dendritic, but not axonal, processes. These data suggest a novel morphogenetic role for neuropoietic cytokines.

There is substantial evidence that the trophic and differentiation-inducing effects of neuropoietic cytokines are mediated by the gp130/stat pathway, acting either alone or sometimes in conjunction with other cascades (Ip and Yancopoulous, 1996; Segal and Greenberg, 1996). In contrast, little is known about the signaling mechanisms that are involved in their regressive effects on neurons. In fact, the only previously described regressive activity of this class of growth factors on neurons has been the induction of cell death in immature sympathetic cells (Kessler et al., 1993; Kotzbauer et al., 1994). This study examines the pathway mediating cytokine-induced dendritic retraction and provides evidence that it too involves gp130 and stat3. Thus, in contrast to the neurotrophins that use two distinct classes of receptors to influence sympathetic neurons (Carter and Lewin, 1997), the neuropoietic cytokines appear to use a single pathway to influence survival, differentiation, and process regression in these cells.

MATERIALS AND METHODS

Materials. Recombinant human OP-1 was isolated from medium conditioned by transfected Chinese hamster ovary cells using S-Sepharose and phenyl-Sepharose chromatography followed by RP-HPLC (Sampath et al., 1992). Recombinant human LIF, IL-11, OSM, and murine monoclonal antibody to gp130 (MAB 228) were purchased from R&D Systems (Minneapolis, MN). CNTF, cardiotrophin-1, and IL-6 were purchased from PeproTech (Rocky Hill, NJ), and soluble IL-6 receptor fragment (sIL-6R) and phosphatidylinositol-phospholipase-C (PI-PLC) were from Sigma (St. Louis, MO).

Tissue culture. Sympathetic neurons dissociated from the superior cervical ganglia of perinatal (embryonic day 21 or 1–2 d postnatal) Holtzman rats (Harlan Sprague Dawley, Rockford, IL) were prepared using previously described methods (Higgins et al., 1991). Cells were plated onto poly-D-lysine-coated coverslips and maintained in a serum-free medium containing β-nerve growth factor (NGF, 100 ng/ml). Cytosine-β-D-arabinofuranoside (1 μM) was added to the medium of all cultures for 48 hr beginning on day 2. Experimental treatments were begun on the fifth or sixth day *in vitro*, after non-neuronal cells had been eliminated.

Morphological analyses. Cellular morphology was assessed by the intracellular injection of fluorescent dyes [4% Lucifer yellow or 5% 5(6)-carboxyfluorescein] and by immunocytochemistry. Cultures were immunostained with monoclonal antibodies (mAbs) previously shown to react selectively with dendrites of sympathetic neurons (Lein et al., 1995; Guo et al., 1997); these included mAbs to MAP2 (AP20, Sigma; SMI-52, Sternberger Immunocytochemicals, Baltimore, MD) and to nonphosphorylated forms of the M and H neurofilament subunits (SMI-32, Sternberger Immunocytochemicals). All antigens were localized by indirect immunofluorescence using previously described procedures (Lein et al., 1995). Process length was assessed using Metamorph Software (Universal Imaging, West Chester, PA).

The cellular distribution of the phosphorylated stat3 was visualized by confocal microscopy after cultures had been immunostained with a rabbit polyclonal antibody (New England Biolabs, Beverly, MA) that specifically reacts with stat3 phosphorylated on Tyr705. Confocal images were collected at a section thickness of 1 μm using a Bio-Rad MRC 1000 laser scanning confocal microscope (Bio-Rad, Hercules, CA).

Western blotting analyses. To examine the expression of cytoskeletal proteins and phosphorylated stat3, sympathetic neurons were plated onto polylysine-coated 35 mm dishes and treated with growth factors for varying amounts of time. Cells were then harvested in 50 mM Tris buffer, pH 7.4, containing 0.1% SDS, 2% 2-mercaptoethanol, and 1 mM EDTA, and homogenized by passaging through a 23 gauge needle at 4°C. Cell extracts were boiled for 5 min and centrifuged at 12,000 × *g* for 15 min. The protein concentration of the various supernatants was determined using the Bradford dye reagent (Bio-Rad). Equal amounts of proteins were analyzed by SDS-PAGE. Subsequently proteins were electrophoretically transferred to a nitrocellulose membrane and probed with mAbs (AP20 or SMI-52) to MAP2, mAb to β-tubulin (a gift from Dr. Robert Hard, University of Buffalo, Buffalo, NY), mAb to the phosphorylated forms of the H and M neurofilament subunits (SMI-31, Sternberger Immunocytochemicals), and polyclonal antiserum to the phosphorylated form of stat3. Detection was accomplished using Chemiluminescent Substrate (Pierce Chemical, Rockford, IL) after sequential treatment with biotinylated goat anti-mouse IgG (HyClone Laboratories, Logan, UT) and horseradish peroxidase-conjugated streptavidin (Amersham, Arlington Heights, IL).

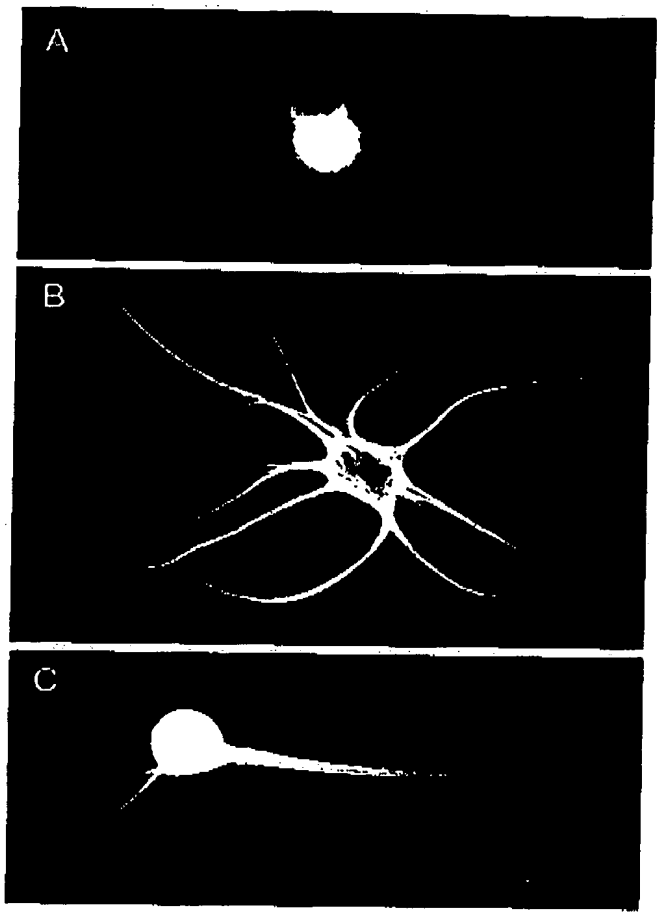

Figure 1. Effects of OP-1 and LIF on sympathetic neurons. *A*, Fluorescence micrograph of a sympathetic neuron that was grown under control conditions before being immunostained with a mAb (SMI52) to MAP2. Such cells typically lacked dendrites, whereas cells that had been treated with OP-1 (50 ng/ml) for 15 d (*B*) had complex arbors. *C*, The size of the dendritic arbor was reduced when cells that had initially been treated with OP-1 for 12 d were treated for an additional 3 d with the combination of LIF (10 ng/ml) and OP-1. Scale bar, 50 μm.

RESULTS

LIF and CNTF induce dendritic retraction

Sympathetic neurons were maintained in a serum-free medium and experimental treatments were begun on the fifth day *in vitro*, after non-neuronal cells had been eliminated. In agreement with previous observations (Lein et al., 1995), it was found that OP-1 induces dendritic growth in these cells (Fig. 1) and that the number of dendrites formed during a 2 week exposure to OP-1 (6.8 ± 0.4 dendrites/cell) closely approximates the number (6.9

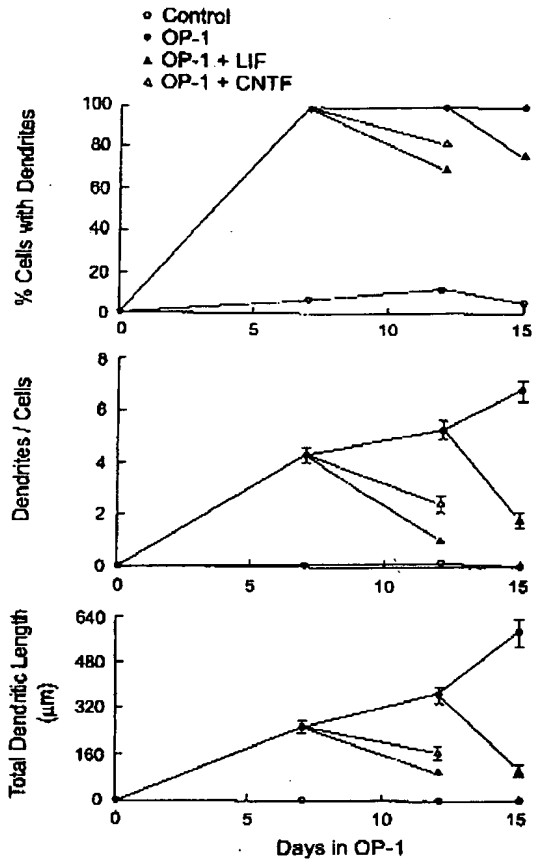

Figure 2. Time course of LIF- and CNTF-induced dendritic retraction in sympathetic neurons. Sympathetic neurons were plated onto polylysine-coated coverslips, and non-neuronal cells were eliminated by a 2 d exposure to an antimitotic agent. Cultures were then continuously treated with OP-1 (50 ng/ml). On day 7 or 12, some OP-1-treated cultures were also exposed to either LIF (30 ng/ml) or CNTF (30 ng/ml) for 3–5 d. Cellular morphology was analyzed after cultures had been immunostained with an mAb (SMI-52) to MAP2. Data are expressed as mean ± SEM (n = 30).

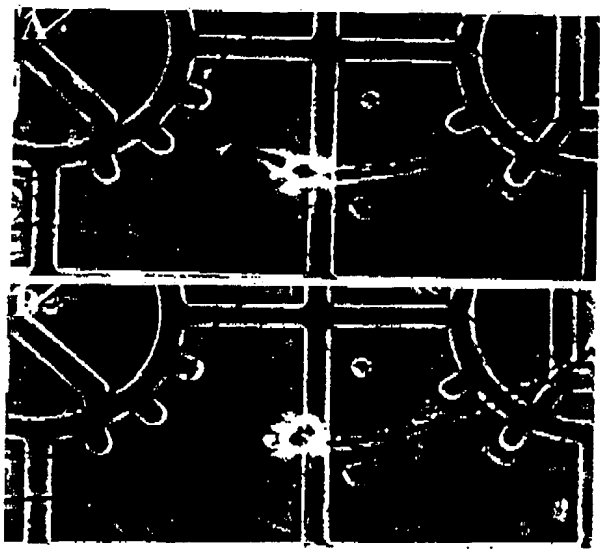

Figure 3. Serial observations of the effects of LIF on neuronal morphology. *A*, Phase-contrast image of a sympathetic neuron's that had been treated with OP-1 (50 ng/ml) for 4 d. One of this neuron's dendrites (*arrowhead*) could be examined in its entirety because it grew through an area where there were only a few axons. The cell was then treated with LIF (10 ng/ml) and OP-1 for 2 d. When the neuron was relocated (*B*), it was found that the dendrite had retracted. In contrast, the density of the axonal plexus increased and new processes appeared (*bottom right* and *left*). Note that the cell shown in *A* has a second, thick dendrite-like process that arises from the right side of the cell and that this process is associated with a bundle of axons. Such fasciculated processes were excluded from our analysis because we could not accurately determine where they ended.

dendrites/cell) arising from sympathetic neurons of 2-week-old rats *in situ* (Snider, 1988). Similarly, the size of the dendritic arbor (total linear length of 590 ± 50 μm/cell) generated during this period *in vitro* was ~75% the size of that observed *in vivo*.

To assess the effects of LIF on existing dendritic processes, neurons were initially exposed to OP-1 for 12 d. Subsequently they were treated for an additional 3 d with either OP-1 alone or with the combination of both LIF and OP-1. On the 15th day, cultures were immunostained with an antibody to MAP2, and it was found that the neurons that had been treated with both LIF and OP-1 had fewer dendrites and smaller arbors than the cells exposed to OP-1 alone (Figs. 1, 2). These data indicate that LIF inhibits the growth of existing dendrites. It was also found that neurons that had been exposed to LIF and OP-1 on days 13–15 had smaller arbors than neurons that had been stained after 12 d of OP-1 treatment, indicating that LIF also causes the retraction of existing processes. The LIF-induced decrease in the size of the arbor reflected a reduction both in the number of primary dendrites (Fig. 2) and in the length of the individual processes (data not shown). Exposure to LIF also caused a 25% decrease in the percentage of cells with dendrites (Fig. 2), indicating that many neurons eliminated all of these processes.

The data in Figures 1 and 2 were obtained from cultures that had been immunostained with antibody to MAP2. However, similar results were obtained when cellular morphology was analyzed by intracellular dye injection or by immunostaining with another dendritic marker, antibody to nonphosphorylated forms of the M and H neurofilament subunits (data not shown). Thus, the effects of LIF reflect actual process elimination rather than the loss of MAP2 from distal dendrites.

LIF-induced dendritic retraction was also observed in cultures of perinatal neurons that had been treated with OP-1 for 4 (Fig. 3), 7 (Fig. 2), and 27 (data not shown) d, and the magnitude of the decrease in the extent of the dendritic arbor (83, 73, and 75%, respectively) was similar to that observed in the aforementioned 12 d cultures (82%). LIF also caused dendritic retraction in cultures of adult sympathetic neurons that had been treated with OP-1 for 7 d (data not shown). Thus, it appears that the effects of LIF on dendritic growth remain relatively constant in nature and magnitude during development.

Serial observations of LIF-treated dendrites

The response to LIF was further characterized by examining its effects on the behavior of individual processes. Sympathetic neurons were plated at low density onto gridded coverslips and then treated with OP-1. Under these conditions, it was often possible to visualize individual dendrites of these neurons in their entirety

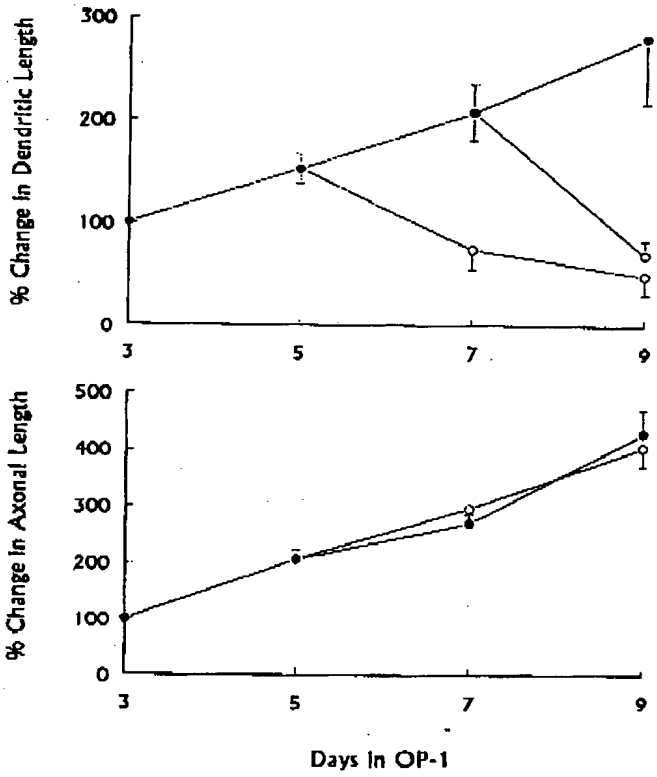

Figure 4. Comparison of changes in dendritic and axonal length during LIF-induced dendritic retraction. Sympathetic neurons were plated onto gridded coverslips and exposed to OP-1 (●, 50 ng/ml) for a total of 9 d. On days 5 and 7, some cultures were also exposed to LIF (○, 30 ng/ml). Identified cells or regions on the grid were photographed daily to allow quantitation of dendritic or axonal growth, respectively. Mean ± SEM (n = 5–12).

(Fig. 3), and our experiments focused on such isolated processes. These processes could be distinguished from axons by light microscope criteria such as their diameter, taper, and branching patterns, and the identity was confirmed in many instances by dye injection.

LIF treatment was begun on the fourth day of OP-1 exposure, and cellular morphology was compared at 1 d intervals. In control cultures treated with OP-1 alone, most dendrites (76%) increased in length, and the remainder (24%) showed no change during the first 24 hr period. In contrast, in cultures treated with the combination of OP-1 and LIF, only 8% of the dendrites elongated during the first day, whereas 73% exhibited no net change in length and 18% underwent retraction. During the second day of exposure to LIF and OP-1, however, the nature of the response began to change: the percentage of dendrites undergoing retraction (43%) rose sharply, whereas the number of elongating dendrites (2%) fell further. It thus appears that the initial response to LIF is an arrest of growth and that several days of exposure to LIF are required to induce retraction in most dendrites.

Serial imaging revealed that although cell bodies became rounded as dendrites retracted, the LIF-treated neurons appeared to be healthy (Fig. 3), and cell number remained constant (data not shown). Moreover, it appeared that axons continued to elongate and generate a more elaborate plexus during LIF treatment. To quantify the changes in axonal length, the amount of axonal growth that occurred in selected areas on gridded coverslips was examined. Images were recorded after 3 d of exposure to OP-1 and again after an additional 4 d exposure to OP-1 or the combination of OP-1 and LIF. Axonal growth was observed in both groups, and the rate of increase was equivalent in cultures treated with OP-1 alone or OP-1 plus LIF (Fig. 4). In contrast, the size of the dendritic arbor was significantly reduced in cultures treated with both OP-1 and LIF as compared with cultures treated with OP-1 alone (Fig. 4). It therefore appears that LIF affects sympathetic neurons in a process-specific manner by selectively modifying the growth of dendrites but not axons. Consistent with this hypothesis, it was found that treatment with LIF decreased the expression of MAP2, a protein found primarily in dendrites, without affecting the expression of phosphorylated forms of the neurofilament H and M subunits, which are primarily axonal proteins (Fig. 5), or β-tubulin, which is distributed throughout the cell.

Concentration–effect relationships and comparison of LIF with other cytokines

Dendritic retraction increased with exposure to greater concentrations of LIF (Fig. 6). Maximum dendritic retraction was observed with concentrations between 10 and 30 ng/ml. The IC_{50} value for LIF-induced dendritic retraction was ~0.3 ng/ml, and significant changes in dendritic morphology could be detected with concentrations as low as 0.01 ng/ml.

To determine whether other members of the IL-6 family of cytokines also induce dendritic retraction in sympathetic neurons, we compared the effects of LIF with those of CNTF, IL-6, IL-11, and OSM. CNTF (Fig. 2) and cardiotrophin-1 (data not shown) consistently induced dendritic retraction, and in most experiments their effects were equivalent in magnitude to those

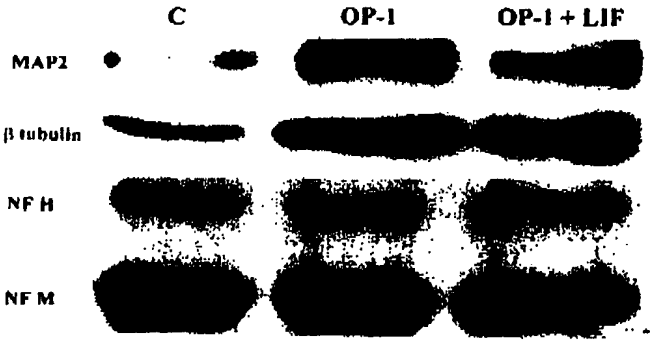

Figure 5. LIF decreases OP-1-induced MAP2 expression. Cultures were exposed to OP-1 (50 ng/ml) for 3 d and then treated with either OP-1 alone or OP-1 + LIF (30 ng/ml) for an additional 4 d. Western blot analysis was performed to examine expression of MAP2 (280 kDa), β-tubulin (53 kDa), and the phosphorylated forms of the H (200 kDa) and M (160 kDa) neurofilament subunits. The chemiluminescent autographs are representative of three experiments that yielded similar results. In these experiments there was 2.5 ± 0.3-fold increase in MAP2 expression in cultures treated with OP-1 ($p < 0.01$ vs control). This was reduced to a 1.4 ± 0.2-fold increase in cultures treated with LIF and OP-1, a value that was not significantly different from the control condition. Similarly, there were no significant changes detected in the expression of phosphorylated forms of the M and H neurofilament subunits under any of the experimental conditions. Exposure to OP-1 produced a 1.9 ± 0.2-fold increase in tubulin expression ($p < 0.01$ vs control), and this change was unaffected by concomitant exposure to LIF (1.8 ± 0.2-fold increase over control).

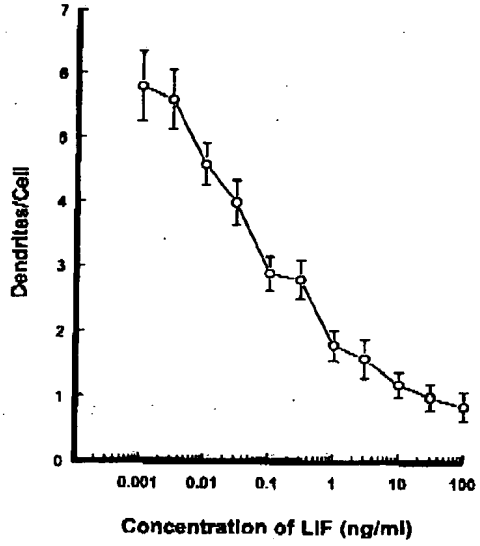

Figure 6. Concentration–effect relationship for LIF-induced dendritic retraction. Cultures of sympathetic neurons were exposed to OP-1 (50 ng/ml) for 3 d and then treated with OP-1 plus various concentrations of LIF for an additional 4 d. The number of dendrites per cell was quantified after cultures had been immunostained with an mAb to the nonphosphorylated forms of the H and M neurofilament subunits. Mean ± SEM (n = 30).

Table 1. Comparison of the effects of members of the IL-6 family of cytokines on dendritic retraction

Growth factor	Dendrites/cell
OP-1	5.6 ± 0.5
OP-1 + LIF	1.1 ± 0.3**
OP-1 + CNTF	1.0 ± 0.2**
OP-1 + IL-6	5.7 ± 0.4
OP-1 + IL-11	5.3 ± 0.4
OP-1 + OSM	4.5 ± 0.4*

Sympathetic cultures were plated onto polylysine-coated coverslips, and nonneuronal cells were eliminated by a 2 d exposure to antimitotic agent. For the next 3 d, cultures were treated with 50 ng/ml OP-1. Subsequently they were exposed to OP-1 alone or in combination with 30 ng/ml LIF, CNTF, IL-6, IL-11, or OSM for 5 d. Cellular morphology was analyzed after cultures had been immunostained with an mAb to the nonphosphorylated forms of the H and M neurofilament subunits. Mean ± SEM (n = 30). *p < 0.05 and **p < 0.001 versus OP-1.

produced by LIF (Table 1). In contrast, IL-6 and IL-11 failed to induce dendritic retraction, and OSM had only weak effects at concentrations up to 30 ng/ml.

Involvement of the gp130 pathway in dendritic retraction

Neuropoietic cytokines exert trophic effects on many types of neural cells, and most of these actions are mediated by the gp130/stat pathway (Ip and Yancopoulos, 1996; Segal and Greenberg, 1996). The finding that several members of this family also caused dendritic retraction suggested that the inhibitory effects of these agents might also be mediated by this pathway. Four experiments were performed to test this hypothesis.

The CNTF receptor complex is a heterotrimer consisting of gp130, the LIF receptor (LIFR), and CNTF receptor α subunit (CNTFR) (Ip and Yancopoulous, 1996; Segal and Greenberg,

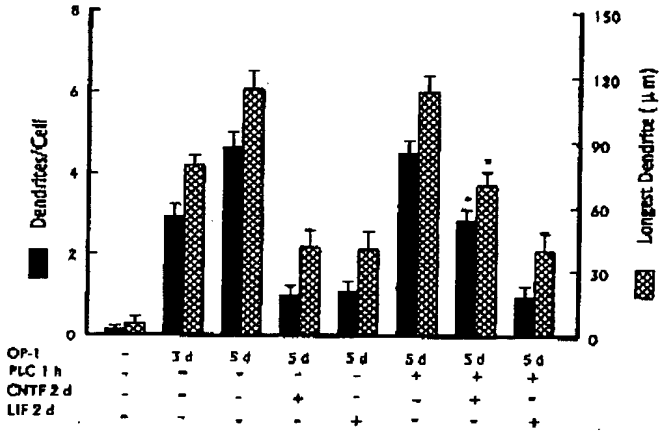

Figure 7. Treatment with PI-PLC reverses CNTF- but not LIF-induced dendritic retraction. Cultures of sympathetic neurons were exposed to OP-1 (50 ng/ml) for 3 d. Subsequently, some cultures were exposed for an additional 2 d to OP-1 combined with CNTF (30 ng/ml) or LIF (30 ng/ml). Other cultures were treated with PI-PLC (1 U/ml) for 1 hr before receiving the aforementioned CNTF or LIF treatments. Cellular morphology was analyzed after immunostaining with an mAb to the nonphosphorylated forms of the H and M neurofilament subunits. Mean ± SEM (n = 30). *p < 0.01 versus OP-1 + CNTF.

1996). The CNTFR specifically confers CNTF responsiveness, and it is linked to the cell membrane via a glycosylphosphatidylinositol bond that can be cleaved by PI-PLC. In contrast, the two subunits (gp130 and LIFR) that are required for responsiveness to LIF are integral membrane proteins that are unaffected by this enzyme. An examination of the effects of PI-PLC treatment on the response of cells to these cytokines revealed that CNTF-induced dendritic retraction was reduced by previous PI-PLC treatment, whereas the response to LIF remained intact (Fig. 7). These results suggest that CNTF-induced dendritic retraction requires the intact CNTF receptor complex.

We next examined the effects of an antibody (MAB 228) to gp130 on cytokine-induced dendritic retraction (Table 2). In these experiments we used a concentration of LIF that produced a 39% decrease in the size of the dendritic arbor. A submaximal concentration of LIF was used because the interaction between MAB 228 and gp130 is influenced by the concentration of the ligand, and so inhibition of the LIF response was more apparent with concentrations near the ED_{50} as compared with higher concentrations. Under these conditions, antibody to gp130 signif-

Table 2. Effects of antibody to gp130 on LIF-induced dendritic retraction

Growth factor	Dendrites/cell
OP-1	4.2 ± 0.3
OP-1 + LIF	2.6 ± 0.3
OP-1 + LIF + anti-gp130	3.5 ± 0.4*
OP-1 + anti-gp130	4.2 ± 0.3

Cultures of sympathetic neurons were treated with OP-1 (100 ng/ml) for 5 d to induce dendritic growth. Beginning on the sixth day, LIF (500 pg/ml), antibody to gp130 (MAB 228, 100 μg/ml), or both agents were added to the OP-1-containing medium of some cultures. Cellular morphology was assessed on the seventh day after immunostaining with antibody to nonphosphorylated forms of the M and H neurofilament subunits. *p < 0.05 compared with OP-1 + LIF treatment.

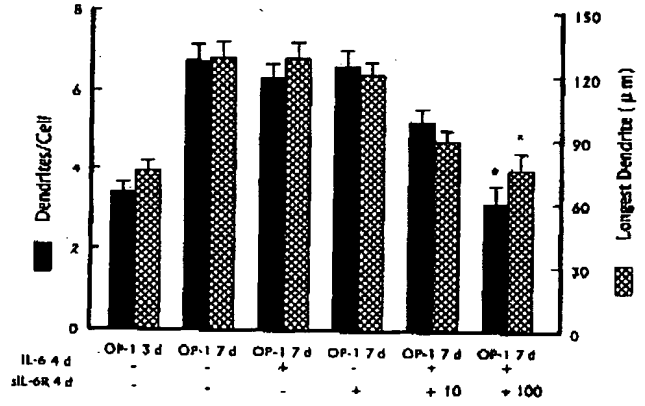

Figure 8. The effects of soluble IL-6 receptor (*sIL-6R*) on dendritic retraction. Cultures of sympathetic neurons were exposed to OP-1 (50 ng/ml) for 3 d. Subsequently, cultures were treated with OP-1 + IL-6 (30 ng/ml), sIL-6R (100 ng/ml), or IL-6 + sIL-6R (10 or 100 ng/ml) for an additional 4 d. Cellular morphology was analyzed after immunostaining with a dendritic-specific antibody (SMI-32). Mean ± SEM ($n = 30$). $^*p < 0.01$ versus OP-1 + IL-6.

icantly attenuated the dendritic retraction induced by either LIF (Table 2) or CNTF (data not shown).

The IL-6 receptor complex is also a heterotrimer that consists of two gp130 subunits and a third subunit called IL-6R (Ip and Yancopoulous, 1996; Gadient and Otten, 1997). Because sympathetic neurons are known to express gp130 in culture, it seemed possible that the failure of these neurons to respond to IL-6 reflected a low level of expression of the IL-6R (Marz et al., 1998). This hypothesis was tested by adding back to cultures a recombinant soluble form of the IL-6R that is known to be capable of forming effective signaling complexes with gp130 (Saito et al., 1991; Marz et al., 1998). Neither IL-6 nor the soluble IL-6R alone had effects on dendritic morphology (Fig. 8). However, the combination of both proteins caused a profound inhibition of dendritic growth, in terms of both the number of dendrites per cell and total dendritic length.

Finally, the effects of neuropoietic cytokines on stat3, a transcription factor involved in gp130 signaling, were examined. Cultures of sympathetic neurons were exposed to OP-1 or the combination f OP-1 with LIF or IL-6 for 20 min. Cellular proteins were then harvested and immunoblotted with an antibody that reacts with the phosphorylated form of stat3. LIF, which is able to induce dendritic retraction, caused significant phosphorylation of stat3, whereas IL-6, which does not affect dendritic growth, was inactive (Fig. 9). Similarly, when cells were immunostained with this antibody, it was found that there was a prominent nuclear translocation of phosphorylated stat3 in LIF- or CNTF-treated cells and that this was absent in control or IL-6-treated cells (Fig. 10).

DISCUSSION

Many molecules have been shown to inhibit the growth of axons (Luo and Raper, 1994; Tessier-Lavigne and Goodman, 1996) and dendrites (Mattson, 1988; LaFont et al., 1994; Guo et al., 1997; McAllister et al., 1997). In contrast, less is known about the agents that cause neurons to eliminate existing processes (Snider and Lichtman, 1996). Our data indicate that LIF and CNTF cause sympathetic neurons to retract most of their dendrites. Cytokine-induced dendritic regression was observed in cultures

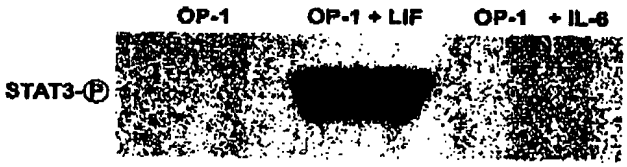

Figure 9. LIF induces phosphorylation of stat3. Cultures of sympathetic neurons were exposed to OP-1 for 3 d and then treated with either LIF (30 ng/ml) or IL-6 (30 ng/ml) for 20 min. Cellular proteins were analyzed by Western blot using an antibody against the phosphorylated forms of stat3. Stat3 had an apparent molecular weight of 92 kDa. The chemiluminescent autographs are representative of three experiments that yielded similar results.

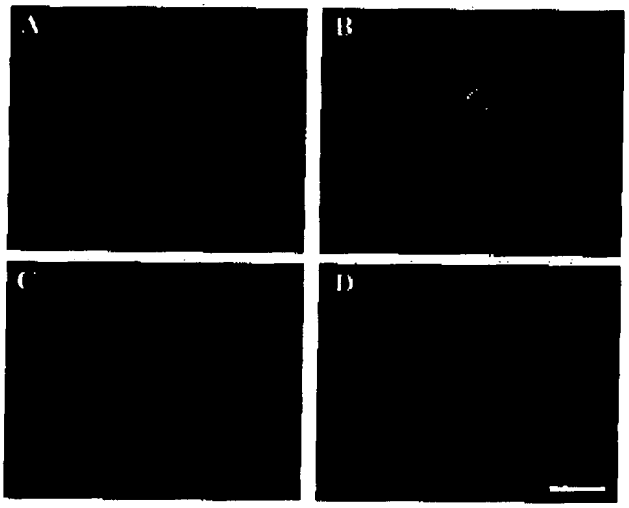

Figure 10. LIF and CNTF induce nuclear translocation of stat3. Cultures of sympathetic neurons were exposed to OP-1 (50 ng/ml) for 3 d (*A*) and then treated for 20 min with 30 ng/ml LIF (*B*), CNTF (*C*), or IL-6 (*D*). Cultures were then fixed and immunostained with an antibody to phosphorylated forms of stat3. The subcellular localization of stat3 was examined in a confocal microscope using 1 μm optical sections. Scale bar, 50 μm.

lacking non-neuronal cells, indicating a direct effect of these agents on neurons. Moreover, dendritic retraction was not accompanied by changes in cell number, axonal growth, or the expression of axonal cytoskeletal elements. These data indicate that LIF and CNTF can modify cell shape in a highly selective manner and that they therefore have the potential to function as morphogens.

Dendritic growth has been studied extensively in sympathetic ganglia (Purves et al., 1988), and its regulation appears to be multifactorial. Previous studies have shown that OP-1 and related bone morphogenetic proteins induce dendritic growth in sympathetic neurons (Lein et al., 1995) and that NGF is required as a cofactor for this activity (Snider, 1988; Lein et al., 1995). In addition, there are at least two classes of molecules, neuropoietic cytokines (Guo et al., 1997) and retinoids (V. Chandrasekaran and D. Higgins, unpublished observations), that can inhibit the initial extension of these processes. The current study suggests an additional level of complexity in the regulation of the morphological development of these cells by demonstrating that there are also agents that can cause dendritic regression. The latter type of interaction could have profound effects on cell shape, and there is

reason to believe that it may occur *in vivo*. Sweat glands secrete a CNTF-like molecule that determines the neurotransmitter phenotype of the sympathetic neurons projecting to this tissue, and the actions of this molecule are mimicked by LIF and CNTF (Patterson and Nawa, 1993; Patterson, 1994; Landis, 1996). Because the concentrations of neuropoietic cytokines required for dendritic retraction are similar to those eliciting changes in the synthesis of neurotransmitters, it is probable that sufficient sweat gland factor reaches the innervating neurons to alter their dendritic morphology. In addition, sympathetic neurons are also exposed to other sources of neuropoietic cytokines during normal development, including cardiotrophin-1 from the heart (Pennica et al., 1996), CNTF from glia (Ip and Yancopoulos, 1996), and an autocrine supply of LIF (Cheng and Patterson, 1997) and IL-6 (Marz et al., 1998).

Cytokine-induced dendritic retraction may also occur as part of the injury response of sympathetic neurons. Axotomy of superior cervical ganglion neurons leads to large and rapid increases in the synthesis of LIF (Banner and Patterson, 1994; Curtis et al., 1994; Sun et al., 1996) and IL-6 (Gadient and Otten, 1997), and the change in LIF expression is sufficient to induce changes in the neurotransmitter status of these neurons (Rao et al., 1993). Axotomy also causes dendritic retraction in sympathetic ganglia (Purves, 1975; Yawo, 1987). It is generally thought that this dendritic atrophy occurs because the injury deprives the neurons of their target-derived trophic factor, NGF (Purves et al., 1988). This idea is largely based on the observations that NGF deprivation causes dendritic atrophy (Ruit et al., 1990) and that administration of NGF reduces some of the effects of axotomy (Purves and Nja, 1976). However, it has not been directly demonstrated that NGF alters the effects of axotomy on dendritic retraction in sympathetic ganglia (Yawo et al., 1987). Our data suggest that there is an alternative or parallel pathway that can cause dendritic retraction. If this is the case, then injury-induced dendritic regression reflects not only separation from the target tissue but also the effects of pro-inflammatory cytokines. This proposition can be tested directly using transgenic mice lacking the LIF gene (Escary et al., 1993), and under these conditions it will be of interest to determine whether axotomy-induced dendritic retraction is also reduced in other neural populations, including spinal motor neurons (Sumner and Watson, 1971).

Dendritic regression occurs in many neurodegenerative diseases (Lambert et al., 1975; Takashima et al., 1989; Flood and Coleman, 1990; Patt et al., 1991; Masliah et al., 1997), and this is often associated with increased synthesis of neuropoietic cytokines (Patterson, 1994; Patterson, 1995; Gadient and Otten, 1997; Murphy et al., 1997). For example, IL-6 is markedly elevated in the nigrostriatal region of Parkinson's patients (Mogi et al., 1994), and it is also found in the β amyloid plaques characteristic of Alzheimer's disease (Bauer et al., 1991; Strauss et al., 1992; Hull et al., 1996). It is possible, therefore, that neuropoietic cytokines contribute to the dendritic atrophy observed in neurodegenerative diseases. In this respect, it is important to note that the effects of neuropoietic cytokines are dominant, i.e., they cause dendritic retraction even in the presence of optimal concentrations of OP-1 and NGF. Moreover, their effects are not surmounted by increasing the concentration of either of these trophic factors (D. Higgins, unpublished data). If this is also true *in vivo*, this would mean that in some degenerative disorders, administration of neurotrophic factors by themselves may fail to reverse the dendritic atrophy and that adjunctive agents that interfere with the synthesis or activity of cytokines may be required (Carlson et al., 1996; Gadient and Otten, 1997).

Neuropoietic cytokines affect sympathetic neurons in a complex manner. They alter the proliferation and survival of these cells (Ernsberger et al., 1989), stimulate axonal sprouting (Thompson and Majithia, 1998), and induce changes in important aspects of their phenotype such as the neurotransmitter status (Patterson and Nawa, 1993; Landis, 1996) and expression of transmitter receptors (Mehler and Kessler, 1997). In addition, neuropoietic cytokines can exert regressive effects on sympathetic neurons by inhibiting dendritic growth and causing resorption of these processes (Guo et al., 1997). At certain stages of development, they also cause cell death (Kessler et al., 1993; Kotzbauer et al., 1994). Thus, neuropoietic cytokines resemble neurotrophins in that they have pleiotropic effects on sympathetic neurons. Neurotrophins use two classes of receptors: the trk proteins that have been found to have primarily trophic effects (Ip and Yancopoulos, 1996) and the p75 receptor that can exert either positive or negative effects, the latter including cell death (Carter and Lewin, 1997). It seemed important, therefore, to determine whether a single signaling pathway mediates both the trophic and regressive effects of the neuropoietic cytokines on sympathetic neurons.

Cultured sympathetic neurons have been shown to express major components of the gp130/stat pathway, including gp130, the LIFR, and the CNTFR (Wong et al., 1995), and strong evidence indicates that this pathway mediates the effects of cytokines on neurotransmitter status (Habecker et al.; 1997; Marz et al., 1998). On the other hand, evidence for the involvement of gp130/stat in regressive actions of these cytokines has derived primarily from the observation that CNTF-induced cell death was prevented by treatment with a phospholipase known to have the capacity to cleave the glycosylphosphatidylinositol linkage that attaches the CNTFR to the membrane (Kessler et al., 1993). Our experiments confirm this previous observation by demonstrating that phospholipase treatment also specifically blocks the effects of CNTF, but not LIF, on dendritic retraction. The fact that a single phospholipase treatment caused only a partial reversal of CNTF effects probably reflects the subsequent reappearance of a new CNTF receptor on the plasma membrane during the next 48 hr *in vitro*, although this was not tested directly. However, subsequent experiments demonstrated that an antibody to gp130 blocks LIF-induced dendritic retraction, that the phosphorylation and nuclear translocation of stat3 precede the morphological changes, and that exposure to soluble IL-6R endows IL-6 with the ability to induce process regression. Because the IL-6R is known to bind to gp130 (Taga et al., 1989; Mackiewicz et al., 1995), these observations strongly suggest that the gp130 pathway is involved in dendritic retraction.

In summary, our data identify a new activity of cytokines that activate the gp130 pathway and suggest that these mediators may function as neural morphogens. In addition, because dendrites are the primary site of synapse formation in vertebrates and because these agents cause their retraction, the data raise the possibility that neuropoietic cytokines could be involved in synapse elimination.

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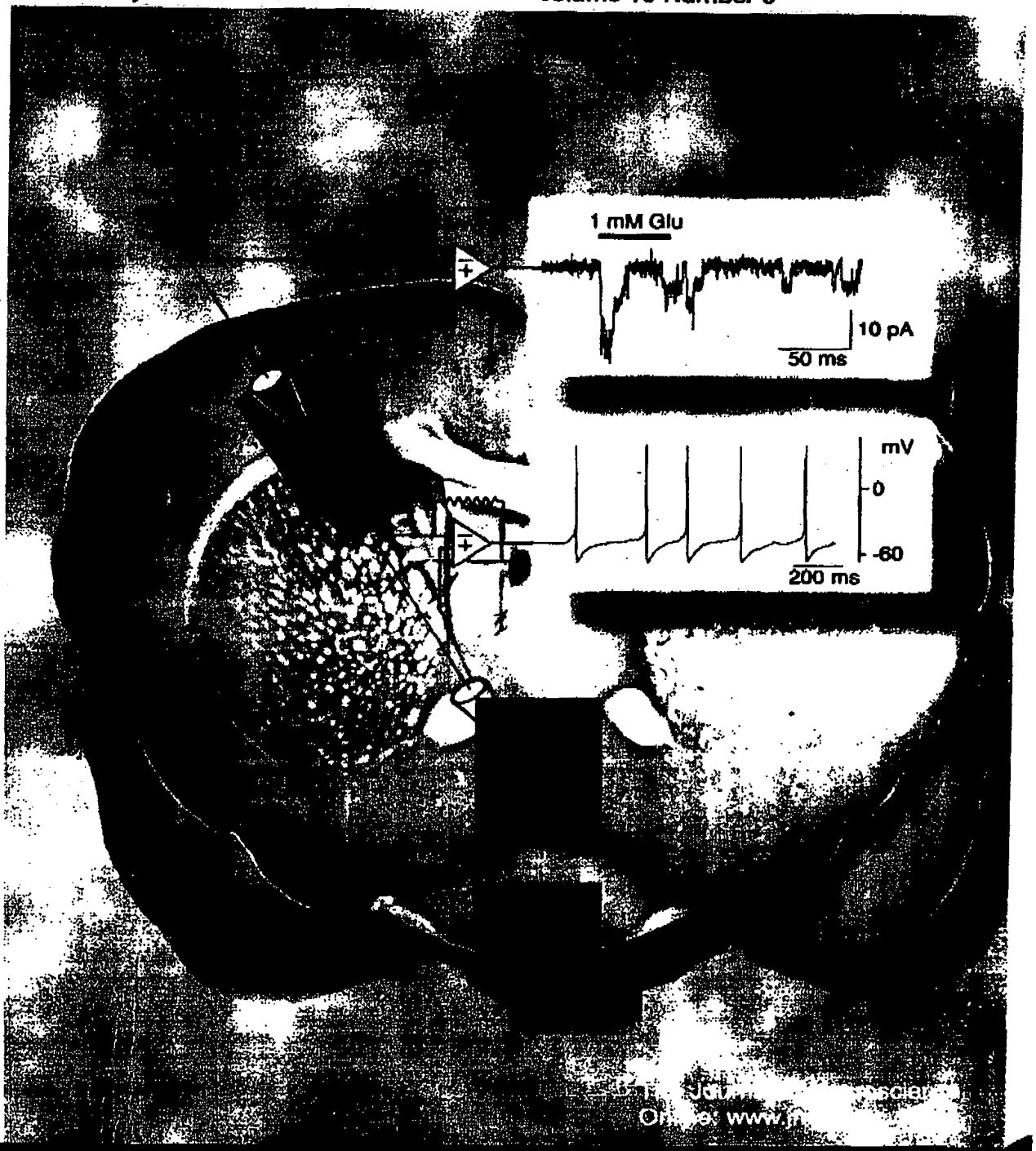

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EXPRESSION OF INTERLEUKIN-6 RECEPTOR, LEUKEMIA INHIBITORY FACTOR RECEPTOR AND GLYCOPROTEIN 130 IN THE MURINE CEREBELLUM AND NEUROPATHOLOGICAL EFFECT OF LEUKEMIA INHIBITORY FACTOR ON CEREBELLAR PURKINJE CELLS

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Abstract—Expression of glycoprotein 130 and the related receptors, including interleukin-6 receptor and leukemia inhibitory factor receptor, was examined in the murine cerebellum at the protein level. Western blot analysis revealed that interleukin-6 receptor, leukemia inhibitory factor receptor and glycoprotein 130 were expressed in the murine cerebellum. Immunoreactivities for interleukin-6 receptor, leukemia inhibitory factor receptor and glycoprotein 130 were strongly localized on the cell body of Purkinje cells, indicating that both interleukin-6 and leukemia inhibitory factor could act directly on Purkinje cells in murine adult mice. The expressions of interleukin-6 receptor, leukemia inhibitory factor receptor and glycoprotein 130 were observed on the cell membranes of Purkinje cells by immunoelectron microscopy. Immunoreactivity for the interleukin-6 receptor was also detected in the cytoplasm of Purkinje cells. Injection of a murine hemopoietic cell line, FDC-P1 cells, transfected with the complementary DNA encoding the leukemia inhibitory factor led to a reduction in calbindin-positive dendrites of the Purkinje cells.

The present results suggest that the leukemia inhibitory factor affects cerebellar functions through Purkinje cells. © 2000 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: dendrites, calbindin, engraft, FDC-P1, cytokines.

Hematopoiesis and immune reactions are regulated by a complex network of stromal interactions and cytokines. These cytokines are produced by various types of cells, either constitutively or inducibly in response to hematologic or immunologic stimulation, and exert varying actions.[1] Although these cytokines show no significant amino acid sequence homology, they share a number of similarities in both structure and function.[16,30] Among those cytokines, interleukin-6 (IL-6), leukemia inhibitory factor (LIF), IL-11, oncostatin M (OSM), ciliary neurotrophic factor (CNTF) and cardiotrophin 1 (CT1) are a family of cytokines sharing many biological effects and playing important roles in reproduction, neural function and bone remodeling, in addition to hemopoiesis and immune responses.[13] Cloning of these receptor genes and reconstitution of the high-affinity receptors for th se cytokines have provided a molecular explanation for their common biological functions;[9,11,27,28,30,38] they act via receptor complexes that contain glycoprotein 130 (gp130), a subunit of the signal transducing protein.[29]

In addition to the mRNA expression of IL-6 and LIF,[25,28] that of their receptor subunits, IL-6 receptor (IL-6R), LIFRβ and gp130, in the rat CNS was demonstrated using an *in situ* hybridization technique; IL-6R mRNA and gp130 mRNA were detected in the granular layer, but LIFRβ mRNA was demonstrated in the Purkinje cells.[28,30,34,36] These findings suggest that the LIFRβ expressed on the Purkinje cells is not functional under normal conditions because the functional receptor for LIF consists of two subunits, LIFRβ and gp130. Recently, Nelson *et al.* showed the expression of IL-6R and gp130 in Purkinje cells using immunohistochemistry.[24] In addition, cerebellar Purkinje cell abnormalities were shown in transgenic mice with cerebral overexpression of IL-6,[5] suggesting that IL-6 exerts its effects on the Purkinje cells through IL-6R and gp130 expressed on those cells. However, the expression of LIFRβ in the cerebellum is still unclear; no cerebellar abnormalities were reported in mice lacking the LIF[4] or LIFRβ,[34] in spite of the expression of LIFRβ mRNA and gp130 in the Purkinje cells.[22,35] The cellular co-localization of LIFRβ with gp130 at the protein level should be analysed to elucidate whether or not the LIFRβ expressed on the Purkinje cells is functional. Such an analysis could help to further characterize the cellular targets of these cytokines and to understand their functional roles. Therefore, in this study, we examined the localization of IL-6R, LIFRβ and gp130 in the cerebellum by immunohistochemistry, and the LIF-induced morphological changes in the murine cerebellum by injecting LIF-secreting cells.

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Abbreviations: BSA, bovine serum albumin; CT1, cardiotrophin 1; CNTF, ciliary neurotrophic factor; GFAP, glial fibrillary acidic protein; GM–CSF, granulocyte-macrophage–colony-stimulating factor; gp130, glycoprotein 130; Ig, immunoglobulin; IL, interleukin; LIF, leukemia inhibitory factor; OSM, oncostatin M; PBS, phosphate-buffered saline.

EXPERIMENTAL PROCEDURES

Animals

Six- to nine-week-old BALB/c and DBA/2 male mice were purchased from Nihon SLC (Shizuoka, Japan). Mice were kept under standardized housing conditions (relative humidity 65–75%, temperature 21–24°C) with a 12-h light/dark cycle (lights on at

06.00) and fed mouse pellets and tap water *ad libitum*. All animal experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1978. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Immunohistochemistry

Tissue specimens were fixed in 4% paraformaldehyde and embedded in an OCT compound. Six-micrometer sections were prepared for immunostaining for IL-6R, LIFRβ and gp130 using an indirect immunoperoxidase technique. Twenty micrometer sections were used for immunostaining for calbindin. In brief, sections were treated with normal goat serum (DAKO, Carpinteria, CA) and incubated with anti-IL-6R, anti-LIFRβ, anti-gp130 (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-calbindin (Chemicon, Temecula, CA) at a dilution of 1:1000 overnight at 4°C. Then, they were incubated with peroxidase-conjugated anti-rabbit immunoglobulin (Ig)G antibody (EnVision Plus, DAKO) for 1 h. After washing, the peroxidase reaction was developed with 0.06% 3-amino-9-ethylcarbazole in 0.05 M Tris buffer (pH 7.7) containing 0.03% hydrogen peroxide. Hematoxylin was used for counterstaining. The following controls were performed: (i) incubation with protein A-purified rabbit IgG instead of the primary antibody; (ii) incubation without the primary antibody or without primary and secondary antibodies; and (iii) incubation with the respective antigenic peptides (1:10 dilution, Santa Cruz Biotechnology) used to produce the primary antibodies prior to staining with them. All controls revealed no labeling.

Immunoelectron microscopy

Mice were fixed by transcardial perfusion of cold periodate-lysine-paraformaldehyde solution, and the brains were removed. These were snap-frozen by *n*-hexane precooled in dry ice-acetone. Frozen sections (6 μm thick) were cut in a cryostat, placed onto glass slides and stained via an indirect method with anti-IL-6R, anti-LIFRβ and anti-gp130, as the first antibody. For the negative control, the sections were incubated with protein A-purified rabbit IgG instead of the primary antibody. After immunostaining, the sections were postfixed with 1% glutaraldehyde and 2% osmium tetroxide solutions, dehydrated in graded ethanol and embedded in Quetol 812 (Nishin EM, Tokyo, Japan). They were detached from the slides after polymerization of the resin. Ultrathin sections of individual samples were cut with an ultramicrotome (Ultracut N, Reichert-Jung, Austria) and examined in a transmission electron microscope (JEM-1200EX, JEOL, Tokyo, Japan).

Western blot analysis

Tissues were immediately placed in RIPA buffer (Boehringer Mannheim, Indianapolis, IN) and homogenized. Twenty microliters of sample buffer (62.5 mM Tris–HCl, pH 7.0, 2.0% sodium dodecylsulphate, 10% glycerol, 0.0025% Bromophenol Blue and 5% 2-mercaptoethanol) was added to each 5 μl of sample and this was boiled for 5 min. Equivalent amounts of protein determined spectrophotometrically from each sample were resolved on 8–16% gradient sodium dodecylsulphate–polyacrylamide gels. Resolved proteins were transferred to nitrocellulose membranes (Amersham, Arlington Heights, IL), using a semidry blot system. Blots were blocked at 4°C in 5% normal donkey serum (Santa Cruz) in phosphate-buffered saline (PBS), pH 7.4 overnight, and then probed for 1 h at room temperature using the anti-IL-6R, anti-LIFRβ and anti-gp130 antibodies diluted to 0.2 μg/ml in PBS containing 0.5% bovine serum albumin (BSA). After washing, the blots were incubated for 1 h at room temperature with peroxidase-labeled donkey anti-rabbit IgG (1:5000; Amersham) in PBS containing 0.5% BSA. Detection of labeled proteins was performed using chemiluminescence (ECL detection reagents, Amersham) according to the manufacturer's instructions.

Construction of the leukemia inhibitory factor-producing cell line

We constructed LIF-producing cells as described previously.[17,22] In brief, a PstI-XhoI fragment of murine LIF cDNA was ligated into the XhoI site of pME18S-neo, which was linearized at the ScaI site. Continuous hemopoietic cell line FDC-P1 cells (FD cells), which are not leukemogenic and are dependent for proliferation *in vitro* on stimulation by granulocyte-macrophage–colony-stimulating factor (GM–CSF) or IL-3, were washed with 20 mM HEPES-buffered saline (pH 7.1) and resuspended at 10^7 cells per ml. Fifty micrograms of linearized plasmid DNA was added to 0.8 ml of the cell suspension in a 0.4 cm electroporation cuvette (Bio-Rad, Hercules, CA). Electroporation was performed using a Gene Pulser (Bio-Rad) at 960 mF and 400 V. After 24 h of incubation, transfectants were selected with G418 (GIBCO-BRL Life Technologies, Tokyo, Japan) at a concentration of 1 mg/ml. After selection for two weeks, stable transfectants were subcloned and analysed for high expression of LIF recombinant proteins by enzyme-linked immunosorbent assay. Cloned lines of FD cells producing high levels of LIF (FD/LIF cells) were grown in complete medium supplemented with 800 μg/ml of G418.

Injection of cells

DBA/2 mice were injected intravenously with 1×10^6 of mock-transfected FD cells (four mice) or 1×10^6 of the highest LIF-producing FD/LIF subline (four mice) weekly. Two months later, mice were killed to perform histological analysis. Tissues were treated as described above.

Quantitative analysis of morphological changes in cerebellar Purkinje cells

Parasagittal sections of the cerebellum, stained immunohistochemically for calbindin, were used to measure the percentage area occupied by calbindin-immunoreactive dendrites in the molecular layer of the cerebellum as described by Walsh *et al.*[33] Digitized images of brain sections were captured directly from a light microscope with a 40× objective magnification. The density of calbindin-immunoreactive dendrites in the molecular layer of the cerebellum was quantified using an image analysis software package (Personal Image Analyzer, ATTO Corp., Tokyo, Japan). The per cent area was calculated by dividing the area of calbindin-immunoreactive dendrites by the area of the molecular layer of the cerebellum being measured. Ten randomly selected regions of the molecular layer of the cerebellum were analysed per slide in four sections per animal, in four animals per condition. Statistical analysis was performed using the Student's *t*-test.

RESULTS

Western blot analysis

To examine the protein expression of IL-6R, LIFRβ and gp130 in the murine cerebellum, its solubilized proteins were analysed by sodium dodecylsulphate–polyacrylamide gel electrophoresis. As shown in Fig. 1, bands corresponding to IL-6R (80,000 mol. wt), LIFRβ (190,000 mol. wt) and gp130 (130,000 mol. wt) were detected in the adult murine cerebellum. These receptors were also detected in the murine cerebrum, although the degree of expression was variable (data not shown). These findings suggest that the proteins of these receptors exit in the adult murine brain.

Immunohistochemical staining

To determine which cells expressed IL-6R, LIFRβ and gp130 in the cerebellum, we performed immunohistochemical staining. As shown in Fig. 2, the Purkinje cells in the cerebellum exhibited intense immunostaining for these receptors. The somata of the Purkinje cells were clearly visible in all of the immunostained sections, but staining for these receptors in the granular cell layer was very faint (Fig. 2A–C). Punctate staining was seen in the molecular layer, which may have been the dendrites of the Purkinje cells (Fig. 2A–C). Fibers within the cerebellar white matter were also stained with the anti-IL-6R antibody, which may represent Purkinje cell axons (data not shown).

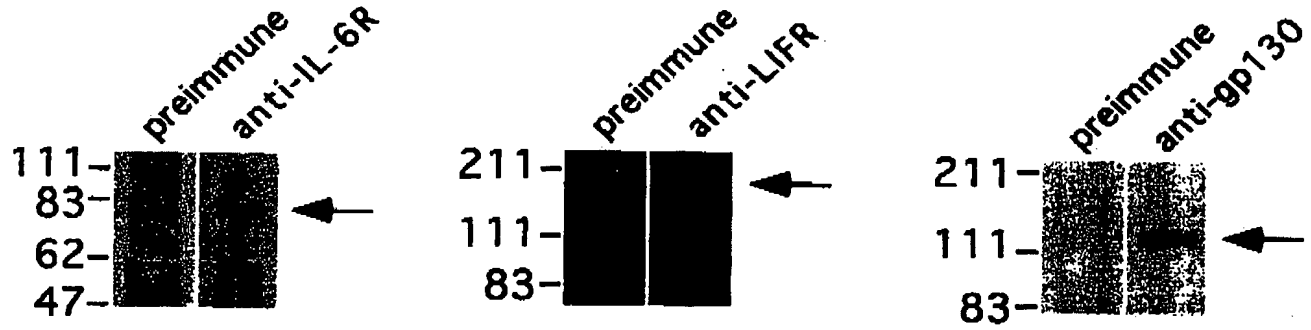

Fig. 1. Western blot of the receptor subunits, IL-6R, LIFR and gp130, in murine cerebellum. Markers are as shown. Ten micrograms of proteins was loaded onto each lane, and western blotting was performed as described in Experimental Procedures. After blotting, the membranes were incubated with polyclonal antibodies against IL-6R, LIFR and gp130. The samples treated with preimmune sera were used as negative controls. Arrows indicate the position of IL-6R, LIFR and gp130, respectively.

Immunoelectron microscopic analysis

To confirm the ultrastructural localization of IL-6R, LIFRβ and gp130 molecules in the Purkinje cells of the cerebellar cortex, we performed immunoelectron microscopic staining using the antibodies against each molecule. The immunoreactive sites of the IL-6R, LIFRβ and gp130 molecules were observed on the cytoplasmic membranes of the Purkinje cells in the cerebellar cortex (Fig. 3A–C). The reactivity of each molecule on the membrane was relatively weak, but significant compared with the negative control staining (Fig. 3D). The immunoreactivity of IL-6R and gp130 on the Purkinje cells was not only on the cell membrane, but also shown in the cisternae of the rough endoplasmic reticulum in the pericytoplasmic region, and in a part of the cytosol with a diffuse pattern. Other organelles in the cells did not show any reactivity with the antibodies. Thus, both IL-6 and LIF may have some effects on cerebellar Purkinje cells in adult mice.

Effects of leukemia inhibitory factor in the cerebellum

To examine the effects of LIF on the murine cerebellum, we injected FD cells or FD/LIF cells intravenously into syngeneic mice. As described by Metcalf,[22] recipients of FD/LIF cells exhibited weight loss [recipients of FD cells (four mice): 22.0 ± 0.5 g, recipients of FD/LIF cells (four mice): 18.5 ± 0.8 g], as well as calcification in heart and skeletal muscles and atrophy of thymus. No macroscopic abnormalities were found in the CNS of either recipient group. Microscopically, a few of the engrafted FD or FD/LIF cells infiltrated to the surrounding area of some microvessels in the brain of both mice. No obvious changes in brain parenchyma were observed in the sections with conventional staining except for the alteration of cerebellar Purkinje cells; atrophic changes were seen in some cerebellar Purkinje cells, but neovascularization, which is a striking feature in glial fibrillary acidic protein (GFAP)-IL-6 transgenic mice,[5] was not detected (Fig. 4). To examine the alterations of the Purkinje cells, immunostaining for calbindin was performed on the murine cerebellum. As shown in Fig. 4, calbindin-immunoreactive dendritic processes of the Purkinje cells of the recipients of FD/LIF cells showed reduced calbindin-immunoreactive dendrites, compared with those of FD cells. In addition, the percentage area occupied by calbindin-immunoreactive dendrites in the molecular layer of the cerebellum in FD-injected and FD/LIF-injected mice was measured. The per cent area occupied by calbindin-immunoreactive dendrites in FD/LIF-injected mice was significantly lower than that measured in FD-injected mice, as shown in Table 1. These findings suggest that LIF plays a role in the alterations of the biochemical nature of cerebellar Purkinje cells, including the reduction of calbindin in the dendrites of Purkinje cells.

DISCUSSION

The IL-6 family of cytokines includes IL-6, LIF, IL-11, OSM, CNTF and CT1, which are known as important mediators of inflammation and immune responses in various tissues including the CNS.[16,30] Recently, it was reported that these cytokines also play a role in neural development.[21] Moreover, some investigators reported that some such cytokines were produced in the CNS of normal adult rats; mRNAs for IL-6, LIF and CNTF, have been detected in the normal brain.[10,14,19,25,28,37] In contrast to the rat, no IL-6 mRNA was detected in the brain of normal mice.[6]

A unique feature of this cytokine family is that the receptors for these cytokines consist of a ligand-specific receptor and a common signal transducer, gp130.[16] This unique receptor system could well explain the functional redundancy of the cytokines. In addition to the ligands, several previous studies have provided information about the mRNA localization of such receptor subunits in the rat brain.[28,31,35,36] However, the cerebellar localization of these receptor subunits is still controversial. Studies using *in situ* hybridization found that the expression of both IL-6R and gp130 was detectable in granular cells of the cerebellum, although LIFRβ was expressed in cerebellar Purkinje cells.[27,31,36] However, if LIF has a functional role through LIFRβ in the cerebellum, both LIFRβ and gp130 should be located in the same cells because the functional receptor for LIF consists of a heterodimer of LIFRβ and gp130.[16] In GFAP-IL-6 transgenic mice, in which IL-6 is overexpressed under the regulatory control of the GFAP gene promotor in the CNS, the dendritic processes of cerebellar Purkinje cells are tortuous and dilated and showed 50% less branching than those in controls,[5] with no abnormalities in granular cells of the cerebellum. This indicates that both IL-6R and gp130 may exist in murine cerebellar Purkinje cells. To address this issue, we examined the localization of IL-6R, LIFRβ and gp130 in the murine cerebellum in the present study, and revealed

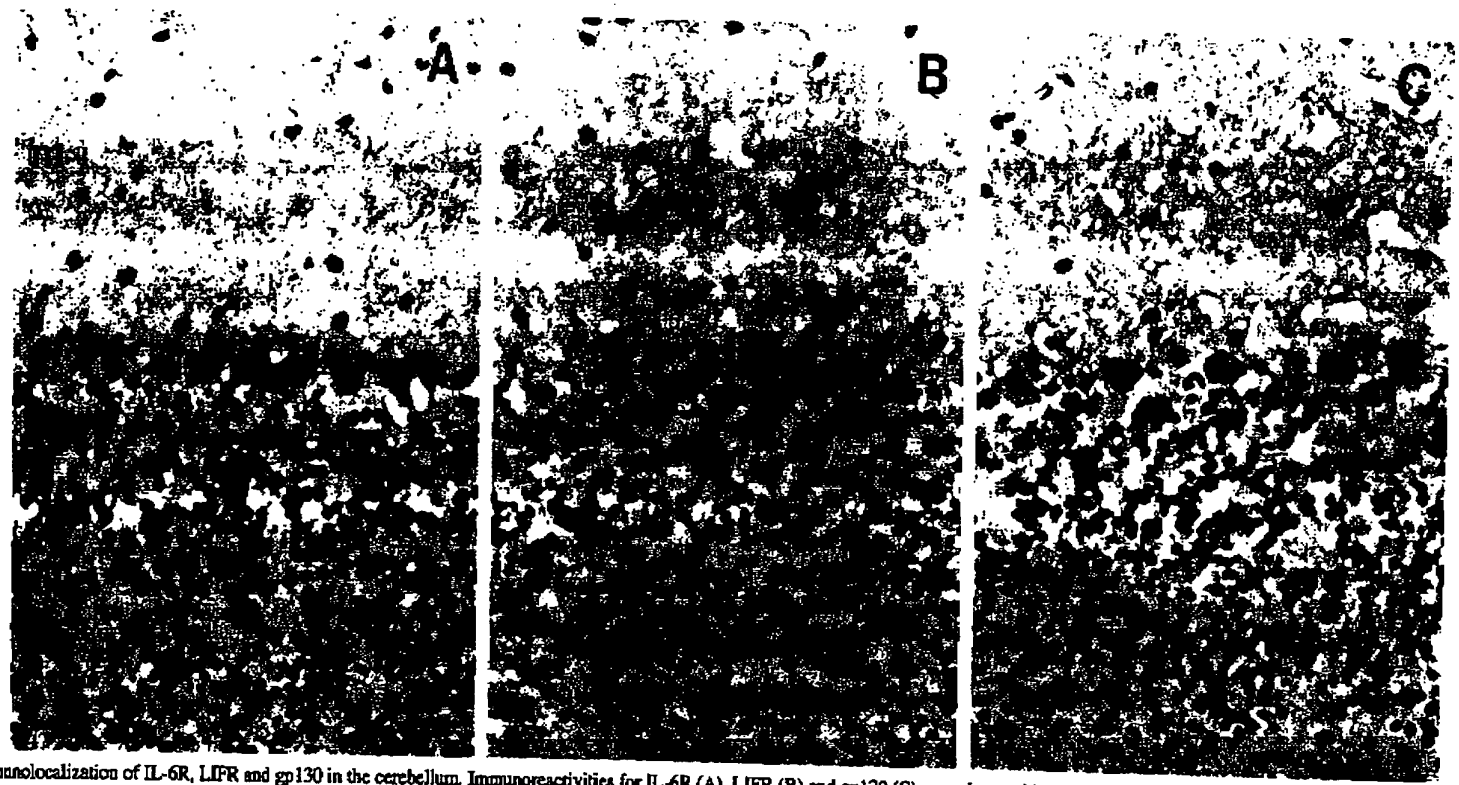

Fig. 2. Immunolocalization of IL-6R, LIFR and gp130 in the cerebellum. Immunoreactivities for IL-6R (A), LIFR (B) and gp130 (C) were detected in the Purkinje cells. IL-6R-positive glia-like cells (A) and gp130-positive granule cells (C) were seen in the granular layer. LIFR was exclusively observed in the Purkinje cells. m, molecular layer; p, Purkinje cell layer; g, granular lyer. Original magnification, ×285.

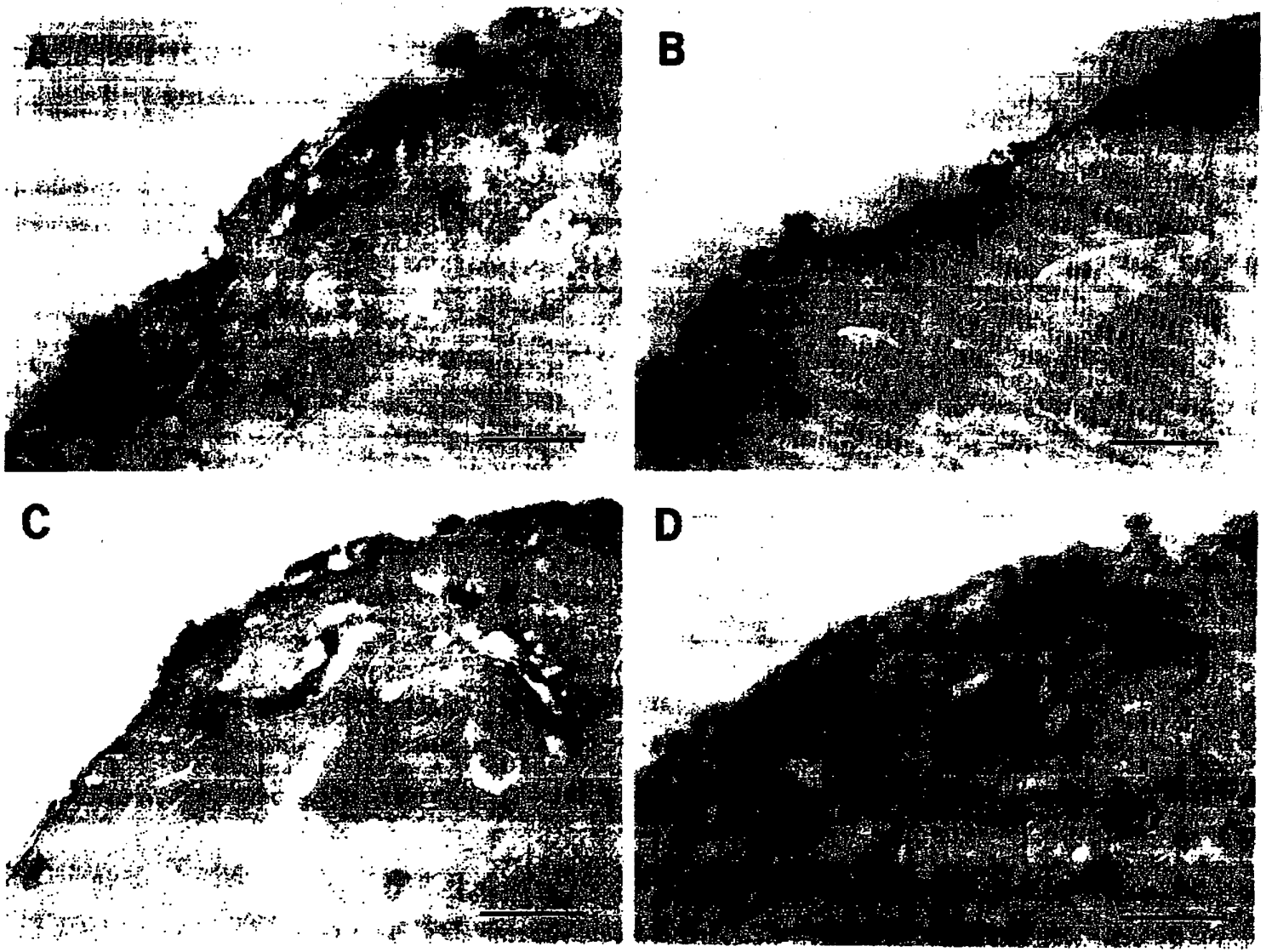

Fig. 3. Representative immunoelectron micrographs of murine cerebellar Purkinje cells stained with anti-IL-6R (A), anti-LIFR (B) and anti-gp130 (C) antibodies. For the negative control, the sections were incubated with protein A-purified rabbit IgG instead of primary antibody (D). Note the immunoreactive cell membrane in each micrograph, and the positively stained rER in (A). Original magnification, ×18,000. Scale bar = 1 mm.

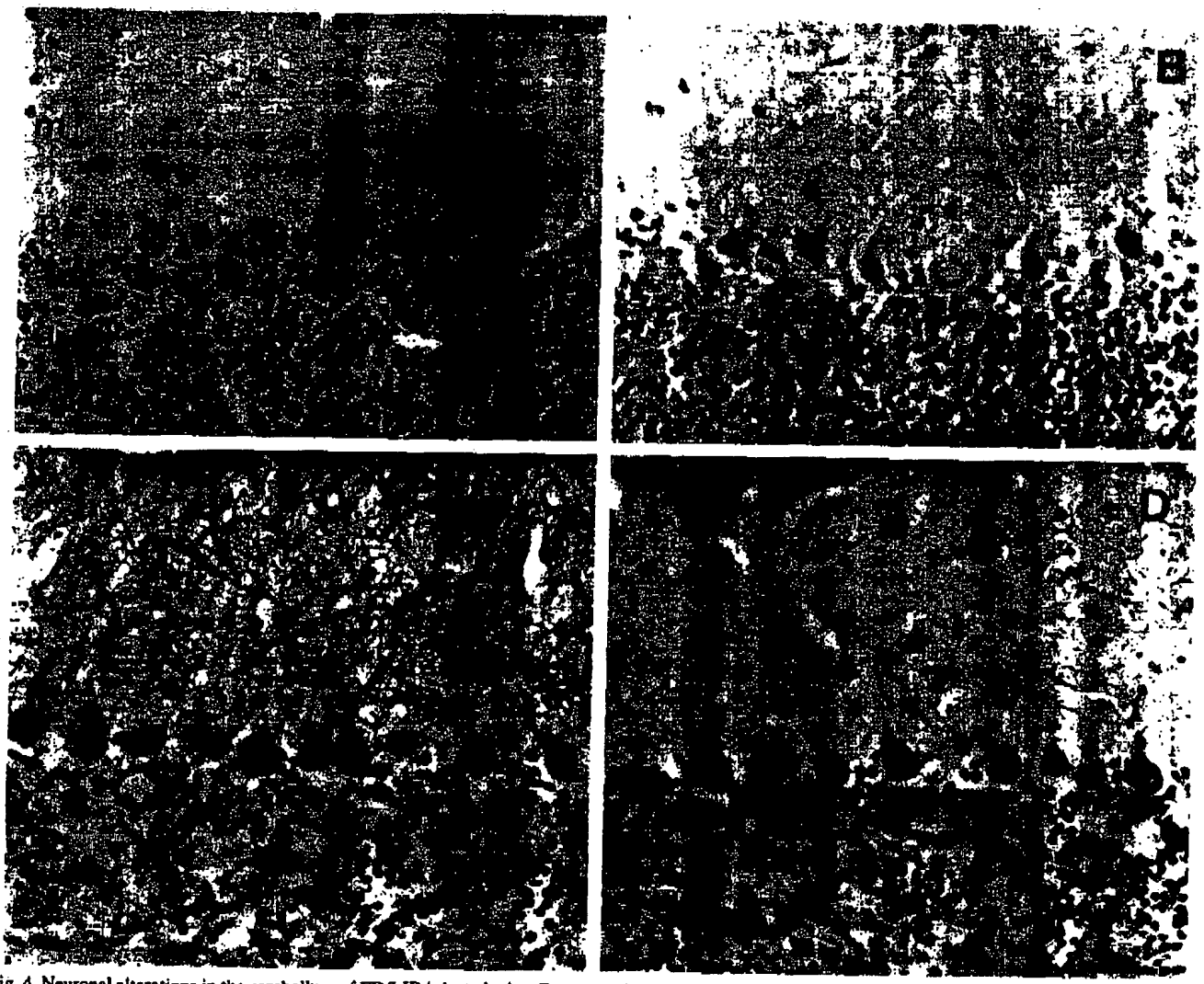

Fig. 4. Neuronal alterations in the cerebellum of FD/LIF-injected mice. Parasagittal sections from mice intravenously injected with FD cells (A, C) or FD/LIF cells (B, D) were stained with hematoxylin and eosin (A, B) and anti-calbindin antibody (C, D) by the indirect method as described in Experimental Procedures. Note that some Purkinje cells in FD/LIF-injected mice (arrowheads) are atrophic (B). Immunostaining using anti-calbindin antibody revealed that the branching of calbindin-positive dendrites of Purkinje cells is reduced by the injection of highly LIF-producing cells (D). m, molecular layer; p, Purkinje cell layer; g, granular layer. Original magnification, ×285.

that the proteins of these receptors were expressed in the Purkinje cells. Recently, Nelson *et al.* also showed that Purkinje cells express high levels of both IL-6R and gp130, suggesting that IL-6 could act directly on Purkinje cells to alter their physiological properties.[24] From these findings, IL-6R, LIFRβ and gp130 are thought to be expressed in cerebellar Purkinje cells, on which their ligands may have some effects. Because it is well known that IL-6 and LIF exhibit some overlapping biological functions in hematopoitic and neuronal cells,[12,16,20,30] the roles of IL-6 and LIF in the development of murine Purkinje cells could be redundant, which may explain for the absence of cerebellar abnormalities in IL-6-deficient or LIFRβ-deficient mice.[18,34]

Table 1. Quantitation of calbindin-immunoreactive dendrite density in the molecular layer of cerebellum of FD- or FD/LIF-injected mice

	% area occupied by calbindin-immunoreactive dendrites
FD-injected mice ($n = 4$)	43.0 ± 5.77
FD/LIF-injected mice ($n = 4$)	7.8 ± 2.77*

The area occupied by the Purkinje cell dendritic tree was measured from parasagittal cerebellar sections of FD- or FD/LIF-injected mice processed for anti-calbindin immunocytochemistry. Data are presented as mean ± S.D.
*$P < 0.001$.

Next, we intravenously injected highly LIF-secreting cells, FD/LIF, into adult mice to examine whether their cerebellar alterations mimic those in GFAP-IL-6 transgenic mice, because of the functional similarities of IL-6 and LIF and their common mechanisms of signal transduction.[20] Ectopic expression of LIF in mice injected with FD/LIF reduced the calbindin-positive dendritic processes of the cerebellar Purkinje cells. Although this alteration may reflect either a

reduction in calbindin in the dendrites or the retraction of dendrites, we consider that the latter is more likely for the following reasons. The reduction in dendrite branching is also found in GFAP-IL-6 transgenic mice[5]. LIF causes rat sympathetic neurons to retract their dendrites *in vitro*, and addition of soluble IL-6R to the medium gives IL-6 the ability to cause dendritic regression, as recently reported by Guo *et al.*[12] In addition, the same morphological alterations as Purkinje cells of FD/LIF-injected mice were recently observed in those cells of mice lacking the rev-erbAa orphan receptor without any obvious phenotypes of the behavior.[8]

Neovascularization and astrocytosis, as found in GFAP-IL-6 transgenic mice, were not obvious in FD/LIF-injected mice,[5] in spite of a similar alteration in the Purkinje cell dendrites. Neurologic behavior characterized by running, tremors, ataxia and seizures was also obscure in the latter. These differences between GFAP-IL-6 transgenic mice and FD/LIF-injected mice may result from the developmental stages in which the mice were exposed to excess of these cytokines for the first time; in GFAP-IL-6 mice, exposure of IL-6 starts in the neonatal stages, but in FD/LIF-injected mice, LIF affects mature Purkinje cells in the adult stage. The concentration of such cytokines may also be different between these animals. In addition, the signaling pathways for LIF may be different from those for IL-6. Hermanns *et al.*[13] recently reported that the signaling activity induced by heterodimerization of LIFRβ and gp130 is less pronounced relative to that induced by gp130 homodimerization. This may explain why the FD/LIF-injected mice did not exhibit the severe neurologic symptoms that developed in GFAP-IL-6 mice.

When FD cells and FD/LIF cells were injected intravenously into syngeneic mice, these cells accumulated in the marrow, spleen and lymphoid tissues. There was a marked elevation of circulating LIF levels in the recipients of FD/LIF cells, but no elevation of LIF production or serum LIF levels was seen in control mice injected with FD cells.[22] Thus, the morphological changes in cerebellar Purkinje cells which we observed in FD/LIF-injected mice may either have been the consequence of the direct action of LIF on them or have been mediated indirectly by some LIF-induced products. Although this issue remains to be clarified, we consider the former more likely for the following reasons. First, it has been reported that many cytokines, including IL-1, tumor necrosis factor alpha, insulin-like growth factor 1 and basic fibroblast growth factor, injected intravenously can enter the brain by either the blood–cerebrospinal fluid or blood–brain interfaces,[2,7,23,26,32] while it remains unclear whether LIF injected intravenously can cross the blood–brain barrier. In addition, the engrafted FD/LIF cells infiltrated to the surrounding area of some microvessels. It is possible that circulating LIF produced by FD/LIF cells or LIF produced by infiltrating FD/LIF cells affects cerebellar Purkinje cells. Second, LIF causes the retraction of dendrites of neurons *in vitro*.[12] Third, as shown in the present study, both components of the LIF receptor, LIFRβ and gp130, exist on the plasma membrane of cerebellar Purkinje cells and morphological changes in Purkinje cells in mice injected with LIF-producing cells resemble those of neurons exposed to LIF *in vitro*.[12]

What are the roles of IL-6 and LIF in murine cerebellar development in the embryonic and adult stages? As described above, the cerebellar development of IL-6-deficient or LIFRβ-deficient mice is normal, but targeted disruption of gp130 leads to embryonic lethality.[18,34,38] These findings and our data, taken together, suggest that either IL-6 or LIF, having redundant roles, is necessary for the development of cerebellar Purkinje cells. However, mice in which gp130 was postnatally inactivated do not exhibit cerebellar abnormalities, but show degeneration of the peripheral nerves, myocardial abnormalities, defects in hematopoiesis and immune system, and morphological and functional abnormalities in the liver.[3] From these findings, we propose that IL-6 and LIF may exert double-edged functions on cerebellar Purkinje cells; these cytokines may be necessary for the development of cerebellar Purkinje cells, but large amounts of them may be harmful under normal conditions in adult mice.

CONCLUSIONS

Western blot analysis revealed the presence of IL-6R, LIFR and gp130 in the murine cerebellum. The localization of these receptors on the cell membranes of Purkinje cells was demonstrated by immunohistochemistry. Injection of FDC-P1 cells transfected with the cDNA encoding the LIF led to the reduction in calbindin immunoreactivity of Purkinje cell dendrites. These findings suggest that LIF affects cerebellar functions through Purkinje cells either directly or indirectly.

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